

Applicants: Reba Goodman, et al
U.S. Serial No.: 09/769,902
Filed: January 25, 2001
Page 9

d) Remarks

Reconsideration and allowance of the present application in view of the foregoing amendments and accompanying remarks are respectfully requested.

Claims 1-12 were pending in the subject application. By this Amendment, applicants have amended claims 1 and 9 and added new claims 13-30. Accordingly, upon entry of this Amendment, claims 1-30 will be pending and under examination.

Applicants maintain that amended claims 1 and 9 and new claims 13-30 raise no issue of new matter and are fully supported by the specification as filed.

Support for amended claim 1 may be found inter alia in the specification, as originally filed, on page 4, lines 5-12, articles submitted by applicants in the May 24, 2003 Amendment, and articles attached hereto as **Exhibits A-C**, which show that gene therapy could be used to treat certain genetic diseases selected from the group consisting of diabetes, heart disease, and cancer. These articles will be discussed below. Support for amended claim 9 may be found inter alia in the specification, as originally filed, on page 4, lines 13-21, and the aforementioned articles. Support for new claim 13 may be found inter alia in the specification, as originally filed, on page 4, lines 13-21; page 8, line 23 through page 9 line 30; and page 15, line 16 through page 16, line 5. Support for new claim 14 may be found inter alia in the specification, as originally filed, on page 4, lines 5-13. Support of new claim 15 may be found inter alia in the specification, as originally filed, on

Applicants: Reba Goodman, et al
U.S. Serial No.: 09/769,902
Filed: January 25, 2001
Page 10

page 6, lines 14-17. Support for new claim 16 may be found inter alia in the specification, as originally filed, on page 6, lines 14-17. Support for new claim 17 may be found inter alia in the specification, as originally filed, on page 6, lines 14-17. Support for new claim 18 may be found inter alia in the specification, as originally filed, on page 6, lines 18-21. Support of new claim 19 may be found inter alia in the specification, as originally filed, on page 6, lines 18-21. Support for new claim 20 may be found inter alia in the specification, as originally filed, on page 6, lines 18-21. Support for new claim 21 may be found inter alia in the specification, as originally filed, on page 6, lines 22-24. Support for new claim 22 may be found inter alia in the specification, as originally filed, on page 4, lines 13-21; page 8, line 23 through page 9 line 30; and page 15, line 16 through page 16, line 5. Support for new claim 23 may be found inter alia in the specification, as originally filed, on page 4, lines 5-13. Support of new claim 24 may be found inter alia in the specification, as originally filed, on page 6, lines 14-17. Support for new claim 25 may be found inter alia in the specification, as originally filed, on page 6, lines 14-17. Support for new claim 26 may be found inter alia in the specification, as originally filed, on page 6, lines 14-17. Support for new claim 27 may be found inter alia in the specification, as originally filed, on page 6, lines 18-21. Support of new claim 28 may be found inter alia in the specification, as originally filed, on page 6, lines 18-21. Support for new claim 29 may be found inter alia in the specification, as originally filed, on page 6, lines 18-21. Support for new claim 30 may be found inter alia in the specification, as originally filed, on page 6, lines 22-24.

In the Advisory Action issued June 16, 2003, the Examiner stated

Applicants: Reba Goodman, et al
U.S. Serial No.: 09/769,902
Filed: January 25, 2001
Page 11

that applicant's reply to the February 24, 2003 Final Office Action has overcome the following rejection(s); rejection of claims 1-12 under 35 U.S.C. §112, first paragraph, as adding new matter and rejection of claims 1-12 under 35 U.S.C. §112, second paragraph.

The Examiner stated that continuation of 5 does not place the application in condition for allowance because the rejection of claims 1-12 failing to meet the enablement requirement of 35 U.S.C. §112, first paragraph was maintained in the Final Office Action mailed February 24, 2003. The Examiner stated that in response, applicant cites several articles and provides a Declaration under 37 C.F.R. §1.132 from Dr. Martin Blank to support enablement for the claimed subject matter. The Examiner stated that although the specification mentions that the claimed method can be used to introduce an exogenous insulin gene, the disclosure does not contemplate the treatment of any specific disease using the method. The Examiner stated that therefore, it is assumed that applicant is claiming a method of which comprises gene therapy of any and all conditions. The Examiner stated that this assumption is supported by applicant's statement on page 14 of Paper No. 11 that, "the specification by mentioning gene therapy, inherently means that it is a method of treating any genetic disease." The Examiner stated that, therefore enablement for the full scope of the claimed subject matter requires enablement for gene therapy of any and all genetic diseases in any and all subjects, wherein a subject is an animal of any species which is in need of therapy.

The Examiner stated that applicant again argues that the references cited by the examiner to establish a prima facie case of non-enablement are outdated and that gene therapy is generally enabled due to several recent advances in the field. The Examiner stated that to support this assertion, applicant cites the following articles:

Jindal et al. (2001) Int. J. Exp. Diabetes Res. 2 :129-138,

Applicants: Reba Goodman, et al
U.S. Serial No.: 09/769,902
Filed: January 25, 2001
Page 12

which teaches delivery of preproinsulin into an immunocompromised mouse using an AAV vector; Ye et al. (1999) Science 283:88-91, which teaches delivery of erythropoietin to mice and a rhesus monkey using an AAV vector system; Edelberg et al. (1998) J. Clin. Invest. 101:337-343, which teaches gene transfer of a b2 adrenergic receptor into a murine neonatal cardiac transplantation model; Edelberg et al. (2001) Heart 86:559-562, which teaches gene transfer of a b2 adrenergic receptor into pig hearts; Rosengart et al. (1999) Ann. Surg. 230:466-470, which teaches a 6 month follow-up of a phase I clinical trail of VEGF gene therapy of coronary artery disease; Navarro et al. (1999) Gene Therapy 6:1884-1892, which teaches gene transfer of reporter genes into neurons using an adenovirus; Park et al. (2000) Blood 96:1173-1176, which teaches gene transfer of human factors VIII and IX into mice using a lentiviral vector; Kon et al. (1999) J. Gene Med. 1:186-194, which teaches gene transfer of insulin into diabetic mice; Steiner et al. (1998) Hum. Gene Ther. 9:747-755, which teaches inhibition of tumor cell growth by expression of a c-myc antisense construct from a retrovirus construct; Zhonghua et al. (2001) The First People's Hospital of Shanghai 30:198-201, which teaches Hsp70 regulated expression of a target gene in a tumor cell; Madio et al. (1998) J. Magn. Reson. 8:101-104, which teaches induction of endogenous hsp70 expression using MRI-guided focused ultrasound; Okano et al. (2001) Bioelectromagnetics 22:408-418, which teaches the effect of static magnetic fields on blood pressure in rabbits; Tofani et al. (2001) Bioelectromagnetics 22:419-428, which teaches the effect of static and ELF magnetic fields on tumor growth and apoptosis; DiCarlo et al. (1998) Bioelectromagnetics 19:498-500, which teaches the effect of electromagnetic fields on anoxic chick embryos; Kapturczak et al. (2001) 1:245-258, which provides a review of gene therapy of type 1 diabetes with an emphasis on AAV gene transfer; and Collateral Therapeutics, Inc. (Public Release, 2001), which teaches that germ cells were unaffected following intracoronary delivery of an adenoviral vector.

The Examiner stated that although some of the teachings contained in the cited art are demonstrative of the slow progress towards effective therapy that is characteristic of the gene therapy art, the art does not support applicant's contention that gene therapy is generally enabled for any and all genetic diseases in any and all subjects.

The Examiner stated that the experiments of Jindal et al. were performed in immunocompromised animals and thus do not account for the immune responses that have hindered development of gene therapy treatments.

The Examiner stated that Ye et al. demonstrates the unpredictability of extending results obtained in one mammalian species to other species of mammals. The Examiner stated that experiments performed in mice showed no diminution in induced EPO expression at 6 months after gene transfer, while induced EPO-expression was undetectable in non-human primates 4 months after gene transfer for unknown reasons.

The Examiner stated that Edelberg et al. (1998) summarizes their findings this way, "these studies demonstrate that the basal rate of the heart can be enhanced by local delivery of exogenous genes which can be used to improve our understanding of the regulation of cardiac automaticity...these investigations also provide an integrated experimental approach for identifying candidate genes and developing local delivery approaches for maximizing/prolonging effects of these candidate genes...the above approaches may eventually be useful in the potential development of both transient molecularly mediated and stable cellular-based cardiac pacemakers". The Examiner stated that Edelberg et al. teaches that the method disclosed therein might one day be useful in the potential development of a therapeutic method. The Examiner stated that likewise, Edelberg et al. (2001) teaches that b2 adrenergic receptor gene transfer into pig hearts transiently increased heart rate and characterizes the findings this way, "these studies showed that

Applicants: Reba Goodman, et al
U.S. Serial No.: 09/769,902
Filed: January 25, 2001
Page 14

the basal rate of the heart can be enhanced by local intravascular delivery of exogenous genes. The Examiner stated that these investigations may provide a foundation for developing novel therapeutics for cardiac chronotropic disorders". The Examiner stated that this is far from teaching an enabled gene therapy method applicable to a genetic disease in any subject.

The Examiner stated that although clinical trial of Rosengart et al. shows some trend toward therapeutic effect, the findings are equivocal due to the small number of patients in the study and, as pointed out by Rosengart et al., "the results are too preliminary to substantiate efficacy". The Examiner stated that given the high degree of unpredictability regarding obtaining therapeutic efficacy using gene therapy approaches, as established in previous office actions, the skilled artisan would not view the teachings of Rosengart et al. as enabling for a method of gene therapy in all subjects.

The Examiner stated that Navarro et al. teaches that adenovirus mediated gene transfer can provide reporter gene expression in neurons *in vivo*. The Examiner stated that, however, expression is clearly reduced over time and Navarro provides no guidance with regard to how the method described therein can be used to effectively treat a disease. The Examiner stated that Navarro et al. teaches only that the vector and method disclosed therein, "offers the possibility of transferring 2 potentially therapeutic genes into neurons within brain areas affected by various neurodegenerative diseases". The Examiner stated that given the high degree of unpredictability regarding obtaining therapeutic efficacy using gene therapy approaches, the skilled artisan would not view the teachings of Navarro et al. as enabling for a method of gene therapy in all subjects.

The Examiner stated that Park et al. teaches gene transfer of human factors VIII and IX into mice using a lentiviral vector. The Examiner stated that, however, in contrast to applicant's

assertion that the teachings support the general enablement of gene therapy for genetic diseases, Park et al. exemplifies many of the problems encountered in developing a gene therapy approach to the treatment of any given disease. The Examiner stated that park teaches that expression of factor VIII was inhibited by an immune response against the protein in immunocompetent mice. The Examiner stated that although factor IX expression was better than factor VIII, Park et al. teaches, "[f]urther improvements in lentiviral vectors will likely be required to achieve therapeutic levels of hFIX at a dose that is considered safe". The Examiner stated that therefore, Park et al. teaches that the vectors available as of August 2000 remained inadequate for predictable gene therapy. The Examiner stated that Park et al. further teaches, "to produce therapeutic levels of coagulation factors, techniques need to be developed to promote cell cycle progression in a safer, nonsurgical manner, such as the use of growth factors". The Examiner stated that therefore, Park et al. teaches that much work remains before gene therapy is enabled for the treatment of hemophilia, let alone any and all genetic conditions in any and all subjects.

The Examiner stated that Kon et al. teaches gene transfer of insulin into diabetic mice. The Examiner stated that although some therapeutic effect is obtained in mice, Kon et al. teach, "the present study suggests that insulin gene transfer into skeletal muscle mediated by naked DMA could provide some therapeutic benefit in diabetes mellitus, further substantial improvements will be required before this approach can be seriously considered for clinical use". The Examiner stated that therefore, the teachings of Kon et al. regarding the therapeutic benefit of the method disclosed therein in the treatment of diabetes mellitus are equivocal and clearly indicate the need for further investigation.

The Examiner stated that although Steiner et al. teaches some therapeutic effect of c-myc antisense expression in mouse tumor

models, Steiner et al. teaches that the findings do not generally support a method of gene therapy of prostate cancer in mammals. The Examiner stated that therefore, the skilled artisan, exemplified by the authors of Steiner et al., would not consider the methods set forth therein as broadly enabled for therapeutic effect in all subjects. The Examiner stated that Zhonghua et al. teaches Hsp70 regulated expression of a reporter gene in a tumor cell. The Examiner stated that, however, Zhonghua et al. does not teach a therapeutic effect and given the high degree of unpredictability regarding obtaining therapeutic efficacy using gene therapy approaches, the skilled artisan would not view the teachings of Navarro et al. as enabling for a method of gene therapy in all subjects and for any and all genetic diseases.

The Examiner stated that Madio et al., Okano et al., Tofani et al. and DiCarlo et al. teach various effects of electromagnetic fields on biological systems. The Examiner stated that the teachings do not, however, contemplate gene therapy and therefore do not address the basis of the enablement rejection, which is the unpredictability of achieving therapeutic effect in the treatment of any and all genetic diseases in an and all subjects using gene therapy.

The Examiner stated that Kapturczak et al. provides a review of gene therapy of type 1 diabetes with an emphasis on AAV gene transfer. The Examiner stated that although Kapturczak et al. is generally optimistic about the future of AAV vectors in gene therapy, the authors also acknowledge that there is much work to be done in order to develop an effective gene therapy for type 1 diabetes. The Examiner stated that Kapturczak et al. teaches, "[a]t present, many questions exist that must first be addressed in animal models" including: which gene or genes will be most effective in preventing and/or delaying islet cell transplant rejection; will genes for allograft rejection prevent recurrent autoimmunity and vice versa; and how safe will islet cell immunomodulatory therapy be in terms of overall

Applicants: Reba Goodman, et al
U.S. Serial No.: 09/769,902
Filed: January 25, 2001
Page 17

physiology. The Examiner stated that therefore Kapturczak et al. teaches that there is much to be learned before gene therapy is enabled for type 1 diabetes in any animal let alone any and all subjects.

The Examiner stated that applicant cites Collateral Therapeutics, Inc., which teaches that germ cells were unaffected following intracoronary delivery of an adenoviral vector. The Examiner stated that therapeutic effect was not demonstrated.

The Examiner stated that taken as a whole, the art of record indicates that, at the time of filing, limited therapeutic effects had been observed in experimental models of a handful of genetic diseases. The Examiner stated that it must be emphasized that the claimed method is not limited to gene therapy of any of the diseases for which some evidence of therapeutic effect is shown. The Examiner stated that the claims encompass gene therapy of many intractable diseases and genetic diseases which comprise multiple genetic components or for which the genetic component is unknown. The Examiner stated that this is pointed out by Kapturczak et al. who teaches that one of the problems encountered in developing gene therapy "was the finding that most diseases are polygenic, involving multiple genetic factors. The Examiner stated that an example is the case of type 1 diabetes where at least 18 separate chromosomal regions have been associated with genetic susceptibility to the disorder". The Examiner stated that therefore, even if vector and promoter systems had been perfected by the time the instant application was filed the skilled artisan would not be able to practice the claimed method the vast majority of genetic diseases without engaging in undue experimentation to overcome the barriers to therapeutic effect that are unique to each disease, such as identifying a therapeutic gene.

The Examiner stated that applicant has submitted a Declaration by Dr. Martin Blank, wherein Dr. Blank states his belief that the teachings of the instant disclosure and relevant art as of the filing date of the application would enable one of ordinary

Applicants: Reba Goodman, et al
U.S. Serial No.: 09/769,902
Filed: January 25, 2001
Page 18

skill in the art to conduct a working *in vivo* experiment to practice the claimed invention such that a sustained therapeutic result is obtained. The Examiner stated that in paragraph 6 of the Declaration, Dr. Blank outlines steps involved in introducing an EMRE containing gene into a patient and applying an electromagnetic field. The Examiner stated that the statements in the Declaration have been fully considered but are not found persuasive because they do not address the general unpredictability of obtaining therapeutic effect using gene therapy techniques. The Examiner stated that is, the method steps set forth summarize what would be readily apparent to one of skill in the art but do not address the intricacies of immune response against vectors and transgenes and problems of promoter shutdown, etc., which have hindered the development of therapeutic methods using gene therapy techniques. The Examiner stated that furthermore, the teachings of the declaration are not enabling for the full scope of the claims which, as pointed out above, encompass a method of gene therapy for any and all genetic diseases.

The Examiner stated that in support of enablement based on *in vitro* data, applicant also points to M.P.E.P. §2164 which states that the Examiner must weigh the evidence for and against correlation and decide whether one skilled in the art would accept the model as reasonably correlating to the condition. The Examiner stated, however, given the vast scope of conditions that applicant purports to treat according to the claimed method, it stands to reason that the limited number of examples provided in the specification and cited art cannot be correlated with the full scope of the claims.

The Examiner stated that applicant again criticizes the art cited by the Examiner, which was published in or before 1997, as not representative of the state of the art at the time of filing. The Examiner stated that, however, as applicant is aware, the Examiner's burden is to establish a *prima facie* case

Applicants: Reba Goodman, et al
U.S. Serial No.: 09/769,902
Filed: January 25, 2001
Page 19

of nonenablement. The Examiner stated that the art cited by the examiner establishes that case. The Examiner stated that although applicant has cited more recent art, the teachings of each of the articles cited by applicant have been considered and an explanation of why the art fails to overcome the rejection has been provided. The Examiner stated that as the teachings of the art of record fail to provide enablement for the full scope of the claimed subject matter, the claims stand rejected under 35 U.S.C. §112, first paragraph.

In response, in an attempt to advance prosecution of the subject application, but without conceding the correctness of the Examiner's position, applicants have amended claims 1 and 9. Claims 1 and 9 have been amended to state that the method is used to treat a genetic disease selected from the group consisting of diabetes, heart disease, and cancer. In the Amendment dated May 24, 2003, applicant cited a number of articles, namely Jindal et al. (2001, Rosengart et al (1999), Kon et al. (1999), and Steiner et al. (1998) which showed that gene therapy could be used to treat different types of diseases, including specifically diabetes, heart disease, and cancer.

In addition to the above articles, the following articles demonstrate that gene therapy can be used to treat cancer and heart disease. In Campbell, et al., "Adenovirus-mediated p16^{INK4} gene transfer significantly suppresses human breast cancer growth," *Cancer Gene Therapy*, (2000) 7:1270-1278, it was demonstrated in mice that a adenovirus-mediated p16^{INK4} gene transfer significantly suppressed human breast cancer growth. This article is attached hereto as **Exhibit A**. Another article, Tomiyasu, et al., "Direct intra-cardiomuscular transfer of beta2-adrenergic receptor gene augments cardiac output in cardiomyopathic hamsters," *Gene Ther*, (2000) 7(24):2087-2093, demonstrated that direct intra-cardiomuscular transfer of beta2-adrenergic receptor gene in cardiomyopathic hamsters significantly elevated stroke volume and cardiac output. This article is attached hereto as **Exhibit B**. Furthermore, the abstract, Zhang, et al., "Adenovirus 5 E1a mediated gene therapy

Applicants: Reba Goodman, et al
U.S. Serial No.: 09/769,902
Filed: January 25, 2001
Page 20

for human ovarian cancer cells *in vitro* and *in vivo*," *Int J Gynecol Cancer*, (2001) 11(1):18-23, demonstrated in mice that by using an adenovirus 5E1a gene transfer, tumor growth was greatly inhibited in the mice. This abstract is attached hereto as **Exhibit C**.

In view of the totality of applicants' submitted articles, applicants respectfully disagree with the Examiner's assertion that gene therapy in general was highly unpredictable at the time of filing of the above-identified application. In addition, applicants assert that the Examiner has not demonstrated that the presently claimed invention is not enabling. In the Office Action issued February 24, 2003, the Examiner stated, "Although gene therapy could in theory be used to treat a wide range of diseases, the art of record shows that potential has not as yet been realized and, for reasons of record, could not be realized based on the teachings of the prior art and instant disclosure without engaging in undue experimentation." In support of these assertions the Examiner cited articles from 1997 or earlier although the above-identified application was filed on January 25, 2001. In response, in Paper No. 16, applicants submitted many articles which were more relevant in time, because they were dated between 1998 and 2001 which showed that gene therapy could be used to treat different types of diseases without undue experimentation. In the years between 1997 (the date of the Examiner's articles) and 2001 (the date of applicants' articles), the level of skill as well as the technology progressed such that one skilled in the art following the present application and other publicly available information would have had a reasonable expectation of success as of applicants' filing date, at least for diabetes, heart disease, and cancer.

In the June 16, 2003 Advisory Action, the Examiner appears to acknowledge that the articles submitted in Paper No. 16 show enablement for certain types of diseases when he says, "It must be emphasized that the claimed method is not limited to gene therapy of any of the diseases for which some evidence of

Applicants: Reba Goodman, et al
U.S. Serial No.: 09/769,902
Filed: January 25, 2001
Page 21

therapeutic effect is shown."

Most of these articles submitted by the applicant in Paper No. 16 show that gene therapy could be used to treat different types of diseases. Although some of these articles submitted stated that more work needs to be done in gene therapy, this does not suggest that gene therapy was highly unpredictable, or even unpredictable, at the time of filing. To the contrary, most of these articles showed that without undue experimentation, therapeutic effects could be achieved for treating different types of genetic diseases, including diabetes, heart disease, and cancer.

Applicants believe that, at the time of filing, gene therapy was not highly unpredictable, or even unpredictable, and thus non-enabling. To the contrary, applicants assert that at the time of filing, the teachings of the articles submitted by the applicants demonstrate enablement for using gene therapy to treat at least diabetes, heart disease and cancer.

Claims 2-8 are dependent on claim 1, either directly or indirectly, and are thus patentable for at least the same reasons that claim 1 is patentable. Claims 10-12 are dependent on claim 9, and are thus patentable for at least the same reasons that claim 9 is patentable.

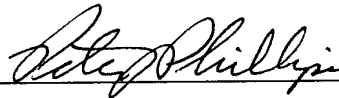
In summary, in light of the remarks and amendments made hereinabove, applicants respectfully request that the Examiner reconsider and withdraw the various grounds of rejection set forth in the February 24, 2003 Office Action.

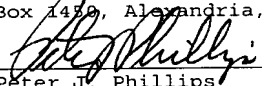
If a telephone interview would be of assistance in advancing prosecution of the subject application, applicants' undersigned attorneys invite the Examiner to telephone them at the number provided below.

Applicants: Reba Goodman, et al
U.S. Serial No.: 09/769,902
Filed: January 25, 2001
Page 22

No fee, other than the enclosed fee of \$972.00 (\$375.00 for filing a RCE, \$465.00 for Petition For A Three-Month Extension Of Time, and \$132.00 for additional claims), is deemed necessary in connection with the filing of the RCE, and this Amendment and Petition For A Three-Month Extension Of Time. However, if any additional fee is required, authorization is hereby given to charge the amount of any such fee to Deposit Account No. 03-3125.

Respectfully submitted,



I hereby certify that this correspondence is being deposited this date with the U.S. Postal Service with sufficient postage as first class mail in an envelope addressed to: Mail Stop RCE, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.	
	5/22/03
Peter J. Phillips	Date
Reg. No. 29,691	

John P. White
Registration No. 28,678
Peter J. Phillips
Registration No. 29,691
Attorneys for Applicants
Cooper & Dunham LLP
1185 Avenue of the Americas
New York, New York 10036
(212) 278-0400



Adenovirus-mediated p16^{INK4} gene transfer significantly suppresses human breast cancer growth

Islay Campbell,¹ Anthony Magliocco,² Terence Moyana,³ Changyu Zheng,⁴ and Jim Xiang¹

Departments of ¹Microbiology and ³Pathology, Saskatoon Cancer Center, University of Saskatchewan, Saskatoon, Saskatchewan, Canada; ²Department of Pathology, University of Calgary, Calgary, Alberta, Canada; and ⁴Gene Therapy Branch, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20892.

The p16^{INK4} tumor suppressor gene encodes a protein that inhibits cyclin-dependent kinase 4, and its homologous deletion is common in human breast cancer. p16^{INK4} gene transfer has been reported to be efficacious in inducing growth inhibition of various human tumors such as brain, lung, prostate, and esophageal cancers. However, the efficiency of the p16^{INK4} gene with regard to growth inhibition of human breast cancer has not been studied extensively. To examine its tumor-suppressive function and its potential in breast cancer gene therapy, the wild-type p16^{INK4} gene was expressed in an adenovirus-mediated gene delivery system and introduced into breast cancer cell lines that do not express p16^{INK4} protein. Expression of the introduced p16^{INK4} blocked tumor cell entry into the S phase of the cell cycle, induced tumor cell apoptosis, and inhibited tumor cell proliferation both *in vitro* and *in vivo*. These results strongly suggest that p16^{INK4} is a tumor suppressor gene and suggest that it has potential utility in breast cancer gene therapy. *Cancer Gene Therapy* (2000) 7, 1270–1278

Key words: p16; adenovirus; apoptosis; breast cancer.

Over the last decade or two, studies on oncogenes and tumor suppressor genes have increasingly provided evidence that cancer develops from the activation of oncogenes and/or the loss or mutation of tumor suppressor genes.^{1,2} Based on this concept, new strategies have been developed rapidly as alternatives to conventional cancer therapy. One of them is cancer gene therapy, whereby insertion of various genes such as the tumor suppressor gene into tumor cells can either restore the normal function of a defective tumor suppressor gene or eliminate the abnormal function of an oncogene product.^{3,4}

p16^{INK4} is a tumor suppressor gene that was identified and cloned from studies of cell senescence⁵ and cell cycle control.⁶ It maps to the chromosome 9p21 region,⁷ which is frequently deleted, mutated, or silenced by promoter methylation in many human tumors.^{8–10} The major transitions of the eukaryotic cell cycle are triggered by cyclin-dependent kinases (CDKs).⁷ CDK4 regulates progression through the G₁ phase of the cell cycle. In normal cells, the retinoblastoma tumor suppressor gene (Rb) regulates cell proliferation by binding and sequestering transcription factors essential for progression to the S phase. These transcription factors are released at the late G₁ phase by phosphorylation of Rb,

thereby allowing cells to enter the S phase. The main function of the CDK4-cyclin D complex is to phosphorylate Rb at late G₁.¹¹ The p16 gene has been biochemically characterized as a protein that specifically binds to and inhibits CDK4 and functions as a negative cell-cycle regulator by controlling the activity of CDK4-cyclin D, leading to regulation of Rb phosphorylation.^{12,13} Because the loss of growth control is a common feature of malignant transformation, it is reasonable to hypothesize that deranged CDK inhibitors such as p16^{INK4} will result in loss of cell growth control. There is now growing evidence that shows that restoration of the p16^{INK4} gene in p16-depleted tumor cells significantly suppresses tumor growth *in vitro* and *in vivo*.^{14–16}

Breast cancer is one of the most common malignancies in North American women, affecting as many as one in eight, and this type of cancer is responsible for as many as one in five cancer-related deaths in women.^{17,18} Aberrant p16^{INK4} expression was reported in 30–60% of breast cancer cell lines,^{19–21} but at a relatively lower frequency in primary breast tumors according to the polymerase chain reaction/single-strand conformation polymorphism method.^{19,20,22,23} However, in a recent study using more controllable immunohistochemical assays, Geradts and Wilson reported a high frequency (49%) of aberrant p16^{INK4} expression in 104 archival breast tumors.²⁴ Their results indicate that aberrant p16^{INK4} expression is much more frequent than was previously thought and may, in fact, be one of the most common abnormalities in human breast cancer.

Received January 27, 2000; accepted May 7, 2000.

Address correspondence and reprint requests to Dr. Jim Xiang, Saskatoon Cancer Center, 20 Campus Drive, Saskatoon, Saskatchewan S7N 4H4, Canada. E-mail address: jxiang@scf.sk.ca

Recombinant adenovirus has facilitated gene transfer into a variety of cell types, both benign and malignant.^{25,26} Adenoviral vectors have a broad host and cell range and high levels of gene expression. The ability to produce large quantities of purified adenovirus with relative ease makes this system very attractive for clinical use. Adenovirus-mediated p16^{INK4} gene transfer as a cancer gene therapy approach has been investigated in several types of human malignancies with p16^{INK4} deletion, including non-small cell lung cancer, esophageal carcinomas, gliomas, and prostate cancer.^{14-16,27} In the present study, we evaluated the relative antitumoral activity of the p16^{INK4} tumor suppressor gene in p16^{INK4}-deleted breast cancer cell lines *in vitro* and *in vivo* by using adenovirus-mediated gene transfer.

MATERIALS AND METHODS

Cell lines, antibodies (Abs), and animals

Various human cell lines were used in this study. These include four breast carcinoma cell lines (MCF-7, MDA-MB-231, BT-549, and T-47D), a cervical epithelioid carcinoma cell line (HeLa), and a lymphoblastic leukemia cell line (MOLT-4). All of these cell lines were obtained from the American Type Culture Collection (Manassas, Va) and maintained in RPMI 1650 medium plus 10% fetal calf sera. The human embryonic kidney cell line 293, transformed with AdV5 E1, was obtained from Microbix Biosystems (Toronto, Ontario, Canada) and maintained in Eagle's minimum essential medium plus 10% fetal calf serum. Various mouse monoclonal Abs recognizing p16^{INK4}, Rb, proliferating cell nuclear antigen (PCNA), and pan-keratin (AE1/AE3/PCK26) were purchased from Pharmingen Canada (Mississauga, Ontario, Canada). BALB/c *nu/nu* mice were purchased from Charles River (Montreal, Quebec, Canada) and maintained in our pathogen-free animal facility at the Saskatoon Cancer Center.

Recombinant adenoviruses

Three recombinant adenoviruses were used in this study, namely AdV-LacZ, AdV-p16, and AdV-pLpA. The adenovirus AdV-LacZ contains the *Escherichia coli* β -galactosidase (β -gal) marker gene under the transcriptional control of the human cytomegalovirus promoter. The adenoviral vector pLpA-p16 was constructed in our laboratory by insertion of the p16^{INK4} cDNA into the adenoviral expression vector pLpA.²⁸ The recombinant adenoviruses AdV-p16 and AdV-pLpA were produced by cotransfection of pLpA-p16 or pLpA DNA with pJM17 DNA in 293 cells.²⁸ Subsequent homologous recombination between the two plasmids (pLpA-p16 or pLpA and pJM17) resulted in the clones of adenoviruses AdV-p16 and AdV-pLpA. Viral seed stocks were then obtained by amplification in 293 cells. Working stocks of the virus were obtained by harvesting 293 cells at 36 hours postinfection with appropriate seed stock. The cell pellet was lysed by three freeze-thaw cycles. Adenovirus was then purified by two rounds of CsCl ultracentrifugation. Purified virus was mixed with 10% glycerol and dialyzed twice against 500 mL of dialysis buffer containing 10 mM tris(hydroxymethyl)aminomethane/HCl (pH 7.5), 1 mM MgCl₂, and 10% glycerol at 4°C for 6 hours. Purified virus was aliquoted and stored at -80°C until use.

Western blot analysis

Tumor cells from one 10-cm dish with or without adenoviral infection were scraped off using a rubber policeman with 300 μ L of extraction buffer containing 125 mM tris(hydroxymethyl)aminomethane, 0.05% sodium dodecyl sulfate, and 10% β -mercaptoethanol. Cell extracts were harvested. To remove cellular debris, cell extracts were centrifuged at 1000 \times g for 5 minutes. The supernatant containing protein samples (30 μ g) was electrophoresed through 15% gels and transferred onto nitrocellulose papers; blots were incubated with various mouse monoclonal Abs (1 μ g/mL) recognizing p16^{INK4}, Rb, and PCNA molecules as controls, respectively. For detection of Rb protein, protein samples were electrophoresed through 8% gels. Blots were washed with phosphate-buffered saline (PBS) containing 0.1% Tween 20 and further incubated with horseradish peroxidase-conjugated goat anti-mouse Ab. Detection was accomplished by using enhanced chemiluminescence reagent according to the manufacturer's protocol (New England Nuclear Life Science Products, Boston, Mass).

Infection of tumor cells in vitro with AdV-LacZ adenovirus

The *in vitro* ability of adenovirus AdV-LacZ to infect various tumor cell lines was assessed. Briefly, four breast cancer cell lines were seeded onto a triplicate of 96-well tissue culture plates at a concentration of 0.5×10^5 cells/well. Serial dilutions of AdV-LacZ stock (2×10^{10} plaque-forming units (PFU)/mL) were added to form different multiplicities of infection (MOIs): 1×10^7 (MOI of 200), 0.5×10^7 (MOI of 100), 0.25×10^7 (MOI of 50), 1.25×10^6 (MOI of 25), 0.6×10^6 (MOI of 12), and 0.3×10^6 (MOI of 6). Cells were incubated with virus at 37°C for 2 hours. Media containing virus were removed and replaced with the regular media. Cells were further incubated at 37°C for 24 hours and then analyzed for β -gal expression.²⁸ Briefly, transfected tumor cells were fixed in PBS containing 37% formaldehyde and 25% glutaraldehyde and stained with 5-bromo-4-chloro-3-indolyl β -D-galactoside (Life Technologies, Burlington, Ontario, Canada). Positive cells had a blue color. The mean reading of positive tumor cells from wells in triplicates were taken as the percentage transduction at each MOI.

Immunohistochemical study

The breast cancer cell lines MCF-7, MDA-MB-231, and T-47D (1×10^5 cells in a 60-mm dish) were infected with AdV-p16 at an MOI of 50 PFU/cell, except for MCF-7, which was infected at an MOI of 150 PFU/cell. Tumor cells were harvested at 1 day postinfection. Cell pellets were fixed in 10% neutral-buffered formalin and routinely embedded in paraffin. Sections were deparaffinized in xylene and rehydrated in graded alcohols. Endogenous peroxidase activity was quenched by incubating the slides with 0.3% hydrogen peroxide in methanol for 15 minutes followed by three PBS rinses. The slides were incubated with normal rabbit sera to reduce any nonspecific staining. For examination of p16 expression, the slides were incubated with mouse anti-p16 Ab and anti-pan-keratin Ab as a control at 10 μ g/mL overnight at 4°C. The slides were washed and subsequently incubated with biotinylated goat anti-mouse immunoglobulin G Ab at 10 μ g/mL. For examination of apoptosis, an apoptosis (APO)-bromodeoxyuridine (BRDU) kit (Phoenix Flow Systems, San Diego, Calif) was used. Briefly, the slides were incubated with the DNA labeling solution containing the terminal deoxynucleotidyl transferase enzyme and bromo (Br)-deoxyuridine triphosphate (dUTP) according



to the manufacturer's protocol. The slides were washed and then incubated with the Ab solution containing the biotin-anti-Br-dUTP Ab. These slides were washed three times with PBS and then incubated with avidin-biotin-peroxidase complex reagents for 30 minutes. The peroxidase activity was developed with freshly prepared 0.06% 3',3'-diaminobenzidine containing 0.1% hydrogen peroxide. Hematoxylin was used as the counterstain.

In vitro cell growth and flow cytometry analysis after Adv-p16 infection

To study the inhibition of cell growth *in vitro*, four breast cancer cell lines (5×10^5 cells in a 60-mm dish) were infected with Adv-p16 and Adv-pLpA (as a control) as described above. Culture medium was used for mock infections. Triplicate dishes of each treatment were counted daily for 5 days. The cell viability was determined by trypan blue exclusion. To measure DNA histograms, tumor cells were harvested 3 days after viral infection, resuspended in ice-cold PBS buffer containing 0.3 mM ethylenediaminetetraacetic acid, and fixed by the gradual addition of ice-cold 100% ethanol to a final concentration of 80%. The fixed cells were kept at 4°C for 3 days, pelleted, and resuspended in 1 mL of the above buffer containing 5 µg/mL propidium iodide (Sigma, St. Louis, Mo) and 10 µg/mL ribonuclease. The samples were incubated at 37°C for 30 minutes and then analyzed using a Coulter Epics XL flow cytometer (Coulter, Burlington, Ontario, Canada). The Multiplus AV software package from Phoenix Flow Systems was used for data analysis. Mean peak fluorescence was determined for each histogram.

Tumorigenicity assay

MDA-MB-231 cells were infected with Adv-p16 and Adv-pLpA virus, respectively, at an MOI of 150. An equal amount of cells was treated with medium as a mock infection. At 2 hours postinfection, infected tumor cells (2×10^6 cells in 50 µL of Metrigel per mouse) were injected subcutaneously (s.c.) into the right thigh of each nude mouse. Metrigel, which contains a mixture of laminin, collagen IV, heparan sulfate, and entactin, was purchased from Becton Dickinson Labware (Bedford, Mass). It has been reported that Metrigel was able to significantly enhance human breast tumor growth in nude mice, although the tumor growth rate was not 100%.²⁹ Mice were sacrificed at 2 weeks after tumor inoculation. Tumors were removed for histological analysis.

Intratumoral (i.t.) vaccination with recombinant adenoviruses

MDA-MB-231 tumor cells (2×10^6 cells in 50 µL of Metrigel per mouse) were injected s.c. into the right thighs of mice. When tumors became palpable (~3 mm in diameter), mice were injected i.t. with 2×10^9 PFU of adenoviruses Adv-p16 and Adv-pLpA, respectively. The injection was repeated at day 4. Tumor growth or regression was monitored by measuring tumor diameter using a caliper. Mice were sacrificed at 2 weeks after the initial adenoviral vaccination, and tumors were removed, weighed, and examined histologically.

RESULTS

Expression of endogenous p16^{INK4} and Rb

The MCF-7 and MDA-MB-231 cell lines had homozygously deleted p16^{INK4} genes,²⁰ whereas the T-47D cell

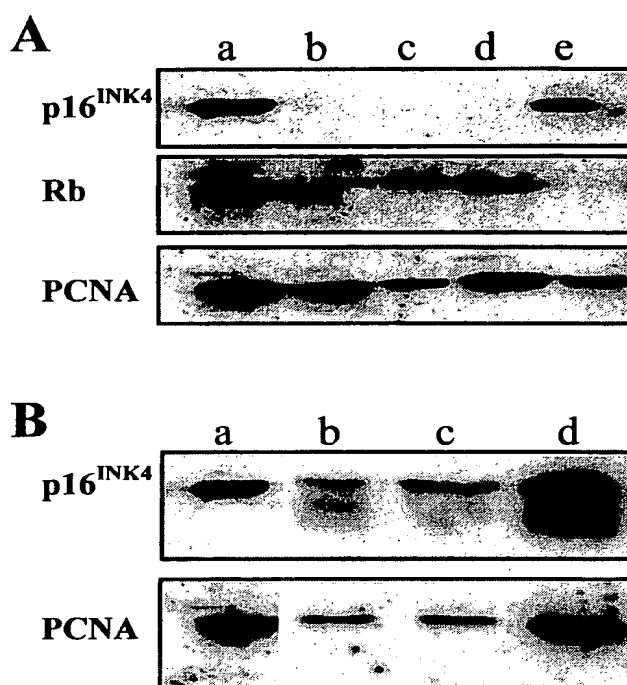


Figure 1. Western blot analysis. **A:** Detection of p16^{INK4} and Rb expression in human tumor cell lines including HeLa and MOLT-4 (lane a), MCF-7 (lane b), MDA-MB-231 (lane c), T-47D (lane d), and BT-549 (lane e). **B:** Detection of p16^{INK4} in a HeLa cell line (lane a) and in Adv-p16-transduced breast tumor cell lines, including MCF-7/Adv-p16 (lane b), MDA-MB-231/Adv-p16 (lane c), and T-47D/Adv-p16 (lane d). In this experiment, the anti-PCNA Ab was used as a control for quantitation of sample protein for each cell line.

line did not express p16^{INK4} protein by DNA methylation.³⁰ All three of the cell lines have a wild-type (wt) Rb gene. The BT-549 cell line, which was selected to act as a control, has a wt p16^{INK4} gene and a mutated Rb gene.³⁰ To confirm the above information, a Western blotting analysis was performed. As shown in Figure 1A (lanes b, c, and d), the MCF-7, MDA-MB-231, and T-47D cell lines showed endogenous expression of Rb but not p16^{INK4} gene, whereas the BT-549 cell line (Fig 1A, lane e) showed endogenous expression of p16^{INK4} but not Rb gene. In this experiment, the protein extracts of HeLa and MOLT-4 cell lines (Fig 1A, lane a) were used as the positive controls for p16^{INK4} and Rb molecules, respectively, and the mouse anti-PCNA Ab was used as a control for quantitation of sample protein of each cell line.

Efficiency of adenoviral infection in breast cancer cells

The infectivity of Adv-LacZ *in vitro* on tumor cells was determined by measuring the percentage of blue cells after cytochemical staining with 5-bromo-4-chloro-3-indolyl β-D-galactoside at 1 day postinfection. A dose-dependent response was noted in each of the cell lines

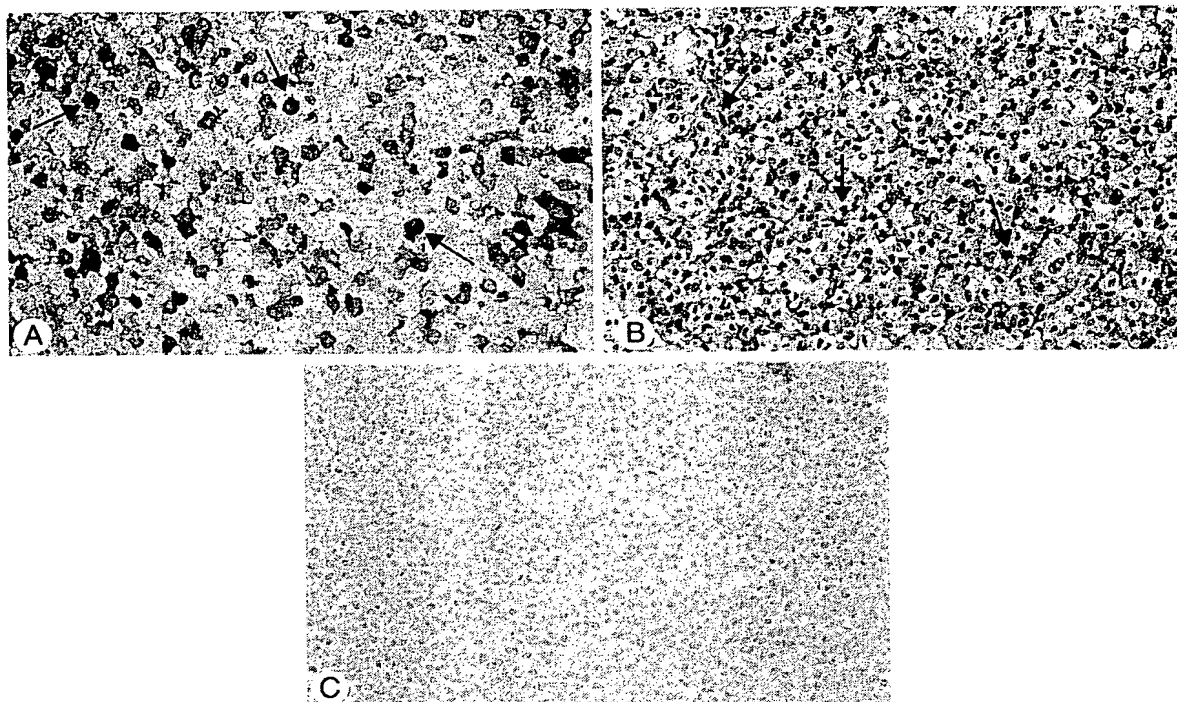


Figure 2. Immunohistochemical study of formalin-fixed, paraffin-embedded MDA-MB-231 breast cancer cells using the anti-p16 and the anti-keratin Abs. **A:** MDA-MB-231 cells infected with AdV-p16 were stained with the mouse anti-p16 Ab. Note the cytoplasmic and nuclear positivity (arrows). **B:** MDA-MB-231 cells were also stained using the mouse anti-keratin Ab as a qualitative control. There is widespread positivity, some of which is indicated by the arrows. **C:** MDA-MB-231 cells without any adenoviral infection were stained with the mouse anti-p16 Ab; no positivity was observed. Magnifications: A, $\times 110$; B, $\times 55$; C, $\times 55$.

tested. Most of the cell lines (MDA-MB-231, T-47D, BT-549, and MCF-7) attained their maximal infectivity (94%, 90%, 81%, and 82%, respectively) at an MOI of 50. The maximal infectivity for MCF-7 was 83% at an MOI of 150. Control cells not exposed to AdV-LacZ did not exhibit any intrinsic β -gal activity or false-positive staining.

Expression of exogenous p16 protein in human breast cancer cells

The strong cytomegalovirus promoter was used to drive p16^{INK4} expression to obtain a high level of expression of this gene. Most of the tumor cell lines were infected with AdV-p16 or AdV-pLpA at an MOI of 50 in the subsequent experiments, except for the MCF-7 cell line, which was infected at an MOI of 150. As shown in Figure 1B (lanes b, c, and d), high levels of exogenous p16^{INK4} protein expression were achieved in the MCF-7, MDA-MB-231, and T-47D cells in Western blots 3 days after AdV-p16 infection. In this experiment, the mouse anti-PCNA Ab was used as a control for quantitation of sample protein. In contrast, cells infected with a control adenovirus, AdV-pLpA, had no detectable increase in the level of p16 expression compared with the level of a control protein PCNA (data not shown). To further

visualize the exogenous expression of p16^{INK4} gene, an immunohistochemical study was performed using the mouse anti-p16 Ab. Three breast cancer cell lines (MCF-7, MDA-MB-231, and T-47D) were infected with AdV-p16. A large amount of exogenous p16^{INK4} expression was detected in both the nuclei and cytoplasm of AdV-p16-infected MDA-MB-231 cells (Fig 2A). In this experiment, the mouse anti-keratin Ab, which detects the cytoplasmic keratins, was used as a control to confirm the quality of this immunohistochemical method (Fig 2B). No endogenous p16^{INK4} expression was seen in this cell line (Fig 2C). The strong and diffuse cytoplasmic expression of p16^{INK4} has also been seen in some human tumor tissues, such as breast and cervical cancers.^{31,32} The expression of exogenous p16^{INK4} was also seen in AdV-p16-infected MCF-7 and T-47D cell lines (data not shown). Subsequently, the effect of the introduced p16^{INK4} protein on the tumor cell lines was examined in the following assays.

Effect of p16^{INK4} protein on breast cancer cell growth

Four breast cancer cell lines (MCF-7, MDA-MB-231, T-47D, and BT-549) were infected with AdV-p16. Triplicate sets of the infected and mock-infected cells were counted every day for 6 days, and the mean cell number

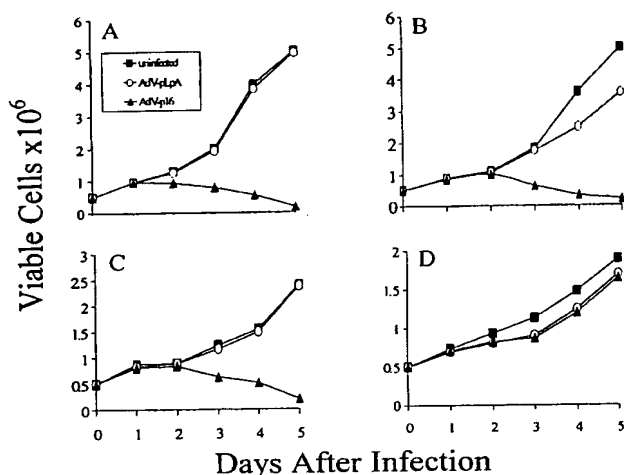


Figure 3. Cell growth curves of the AdV-p16-infected breast cancer cell lines. The MCF-7 (A), MDA-MB-231 (B), T-47D (C), and BT-549 (D) cell lines were incubated at densities of 5×10^5 cells in 60-mm dishes and infected with AdV-p16 or AdV-pLpA. Culture medium alone was used for mock infection. Live cells in triplicate cultures of each cell line for each treatment were counted daily after infection. The curves are plotted from a representative assay. Each point represents the mean of triplicates. The SDs of each point are $<3\%$ of the mean value.

for each day was calculated. As shown in Figure 3, the cell growth curves of four tumor cell lines were similar. However, three cell lines (MCF-7, MDA-MB-231, and T-47D) with aberrant p16^{INK4} and wt Rb gene exhibited marked growth inhibition after AdV-p16 infection, whereas the cell line (BT-549) with wt p16^{INK4} and mutated Rb gene did not show any growth inhibition. All controls representing cell lines with mock infection or infected with AdV-pLpA did not show any significant growth inhibition. These results suggest that tumor cells expressing exogenous p16^{INK4} have an altered exponential growth phase. At 6 days after infection with AdV-p16, the growth rates of the AdV-p16-infected MCF-7, MDA-MB-231, and T-47D cells were inhibited by 94%, 98%, and 90%, respectively, compared with the growth rates of the AdV-pLpA-infected cells. However, the growth rate of BT-549 with endogenous p16^{INK4} infected with AdV-p16 was not inhibited at all.

Cell cycle arrest and apoptosis mediated by AdV-p16

At 3 days after AdV-p16 infection, cells were harvested for cell cycle analysis by flow cytometry. As shown in Figure 4, AdV-p16-mediated expression of p16^{INK4} protein significantly increased 19%, 14%, and 18% of cells in the G₁ phase in the p16^{INK4}-deleted breast cancer cell lines MCF-7, MDA-MB-231, and T-47D, respectively, suggesting the induction of G₁ phase arrest. In addition, two cell lines (MDA-MB-231 and T-47D) also showed 60% and 36% apoptosis that displayed subdiploidy peaks in DNA histograms.³³ In contrast, no G₁ phase

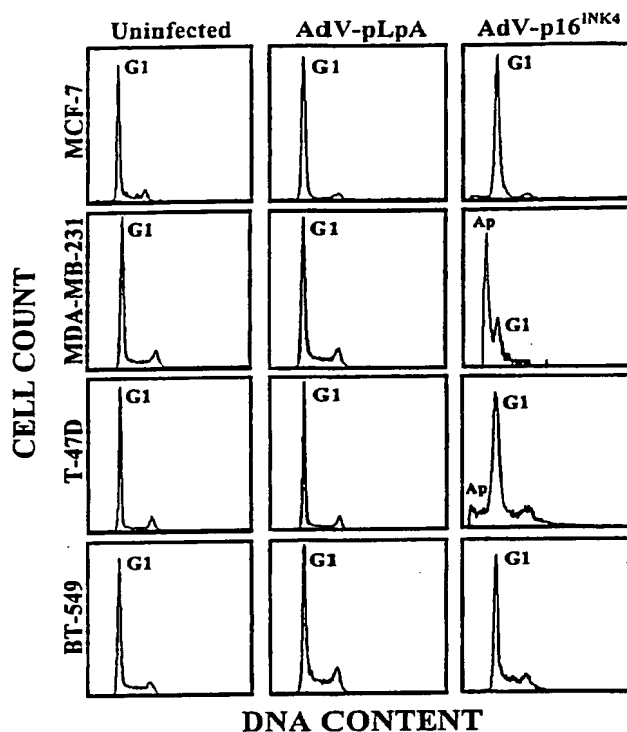


Figure 4. The DNA content of the breast cancer cell lines was analyzed by flow cytometry. MCF-7, MDA-MB-231, T-47D, and BT-549 tumor cells were infected with AdV-p16 and AdV-pLpA, respectively, for 4 days in culture medium. Tumor cell lines were then harvested and stained with propidium iodide for flow cytometric analysis. The uninfected tumor cell lines were used as controls. Apoptosis (Ap) is detected as a fraction of tumor cells with DNA content less than that of G₁ cells.

arrest and apoptosis were seen in p16^{INK4} protein-positive BT-549 with mutated Rb gene. The induced apoptosis was further confirmed in a histochemical study by using the APO-BRDU kit. As shown in Figure 5, scattered apoptosis (arrows) was seen in MDA-MB-231 tumor cells infected with AdV-p16.

Inhibition of tumorigenicity mediated by AdV-p16

To determine whether the exogenous p16^{INK4} expression affects their tumorigenicity, BALB/c *nu/nu* mice were inoculated with MDA-MB-231 tumor cells with and without infection of AdV-p16. As shown in Table 1, AdV-p16-treated tumor cells completely suppressed tumor growth *in vivo*, whereas 70% and 80% of mice that received AdV-pLpA- and medium-treated MDA-MB-231 cells developed tumors, respectively. Our results demonstrated that the tumorigenicity of breast tumor cells was completely inhibited by prior treatment with AdV-p16, indicating that the p16^{INK4} protein may have therapeutic efficacy. The therapeutic potential for AdV-p16 was further examined in a BALB/c *nu/nu* mouse

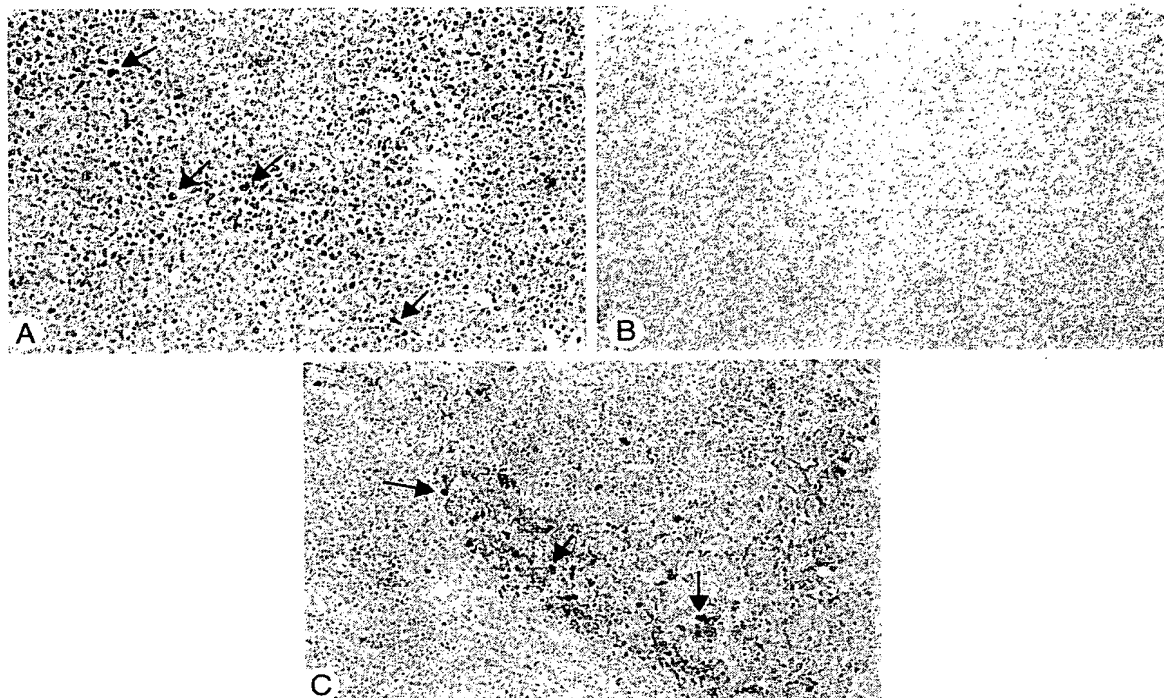


Figure 5. Immunohistochemical study of formalin-fixed, paraffin-embedded MDA-MB-231 tumor cells using the anti-Br-dUTP Ab. **A:** Tumor cells infected with AdV-p16 were pelleted, fixed in formalin, and embedded in paraffin for making sections. The sections were incubated with Br-dUTP and terminal deoxynucleotidyl transferase. After washing with PBS, the sections were incubated with biotin anti-Br-dUTP Ab and then with the avidin-biotin-peroxidase complex reagents. Some of the tumor cells are strongly positive (arrows). **B:** Tumor cells without any adenoviral infection were incubated with biotin anti-Br-dUTP Ab; no positivity was observed. **C:** A human tonsil section was incubated with biotin anti-Br-dUTP Ab and used as a positive control; note the scattered positivity in the lymphoid follicle. Magnifications: $\times 55$ for each figure.

tumor model. As shown in Figure 6 and Table 2, the growth of MDA-MB-231 tumors with treatment of AdV-p16 was significantly inhibited compared with that of tumors that were treated with AdV-pLpA and PBS. The mean weight of AdV-p16-treated tumors was 0.21 g, which is significantly smaller than that of AdV-pLpA- and PBS-treated tumors (1.02 g and 1.23 g, respectively) ($P < .01$). In addition, AdV-pLpA treatment alone has also shown some effect on tumor growth inhibition *in vivo*, although this effect is much milder. As further shown in Figure 7, the AdV-p16-treated tumors showed multiple large areas of necrosis, possibly due to apoptosis induced by exogenous expression of p16^{INK4}, whereas

the AdV-pLpA-treated tumors only displayed small focal areas of necrosis.

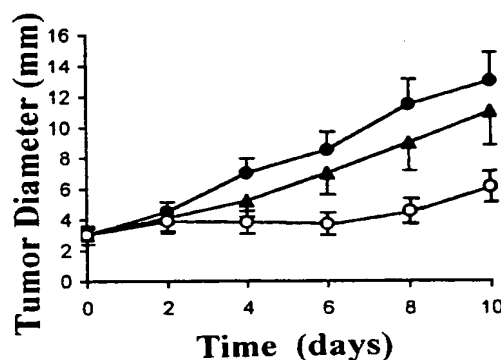


Figure 6. Tumor growth inhibition by i.t. injection of AdV-p16. MDA-MB-231 tumor cells (2×10^6 cells in 50 μ L of Matrigel per mouse) were injected s.c. into the right thighs of nude mice. When tumors became palpable (3 mm in diameter), each mouse was injected i.t. with 2×10^9 PFU AdV-p16 (\circ), 2×10^9 PFU AdV-pLpA (\blacktriangle), or PBS (\bullet), respectively. The injection was repeated once at day 4. Tumor growth or regression was monitored by measuring tumor diameter using a caliper.

Table 1. Tumorigenicity of AdV-p16-infected tumor cells

Tumor cells*	Tumor incidence (%)
MDA-MB-231/AdV-p16	0/10 (0)
MDA-MB-231/AdV-pLpA	7/10 (70)
MDA-MB-231	8/10 (80)

*MDA-MB-231 tumor cells were infected with AdV-p16, AdV-pLpA, and medium, respectively. One day after adenoviral infection, 2×10^6 tumor cells were injected s.c. into each nude mouse. Two weeks after tumor inoculation, tumors were removed and confirmed by histological analysis.



Table 2. Inhibition of established MDA-MB-231 tumors by intratumoral vaccination of AdV-p16

Animal*	Mean tumor weight (g)† ± SD	Tumor incidence (%)
Group I	0.21 ± 0.11‡	8/8 (100)
Group II	1.02 ± 0.25	8/8 (100)
Control	1.23 ± 0.21	8/8 (100)

*There were eight mice in each group. Each nude mouse was injected s.c. with 2×10^6 MDA-MB-231 tumor cells. When tumors became palpable (3 mm in diameter), each mouse in groups I and II was injected i.t. with 2×10^9 PFU of AdV-p16 and AdV-pLpA, respectively. In the control group, each tumor was injected with PBS. Two weeks after injection of AdV-p16, tumors were removed and weighed.

†The mean tumor weight represents the total tumor weight from each group of mice divided by 8.

‡The value in group I is significantly different ($P < .01$) from that in group II and in controls (Student's *t* test).

DISCUSSION

To investigate the efficacy of breast cancer therapy mediated by adenovirus-mediated p16^{INK4} gene transfer, four breast cancer cell lines (MCF-7, MDA-MB-231, T-47D, and BT-549) were selected in this study. These include three tumor cell lines (MCF-7, MDA-MB-231,

and T-47D) with a deficiency in p16^{INK4} expression and one tumor cell line (BT-549) with wt p16^{INK4} but mutated Rb as a control. The aforementioned four cell lines were infected with AdV-p16. Our data showed that they all expressed the exogenous p16^{INK4} after infection, and that the induced expression of p16^{INK4} significantly inhibited their growth rates *in vitro*. It is known that the p16^{INK4} protein is able to inhibit the activity of CDK4, thereby blocking the entry of proliferating cells from G₁ to S phase.³⁴

The mechanism involved in the inhibition of the growth rate mediated by AdV-p16 was examined. The data that we obtained from fluorescence-activated cell sorter analysis showed that expression of exogenous p16^{INK4} blocked tumor cell entry into the S phase of the cell cycle and induced cell apoptosis. The induced apoptosis was further confirmed by immunohistochemical analysis using the APO-BRDU kit. In contrast, no G₁ phase arrest and apoptosis were seen in the BT-549 cell line with wt p16^{INK4} and the mutated Rb gene. These results indicate that wt endogenous Rb expression is a critical determinant of p16^{INK4}-mediated cell cycle arrest and cytotoxicity; they also suggest that the p16^{INK4} protein not only suppresses the growth of tumor cells by mediating G₁ arrest but also induces cell death by apoptosis in tumor cell lines that have wt Rb but do not

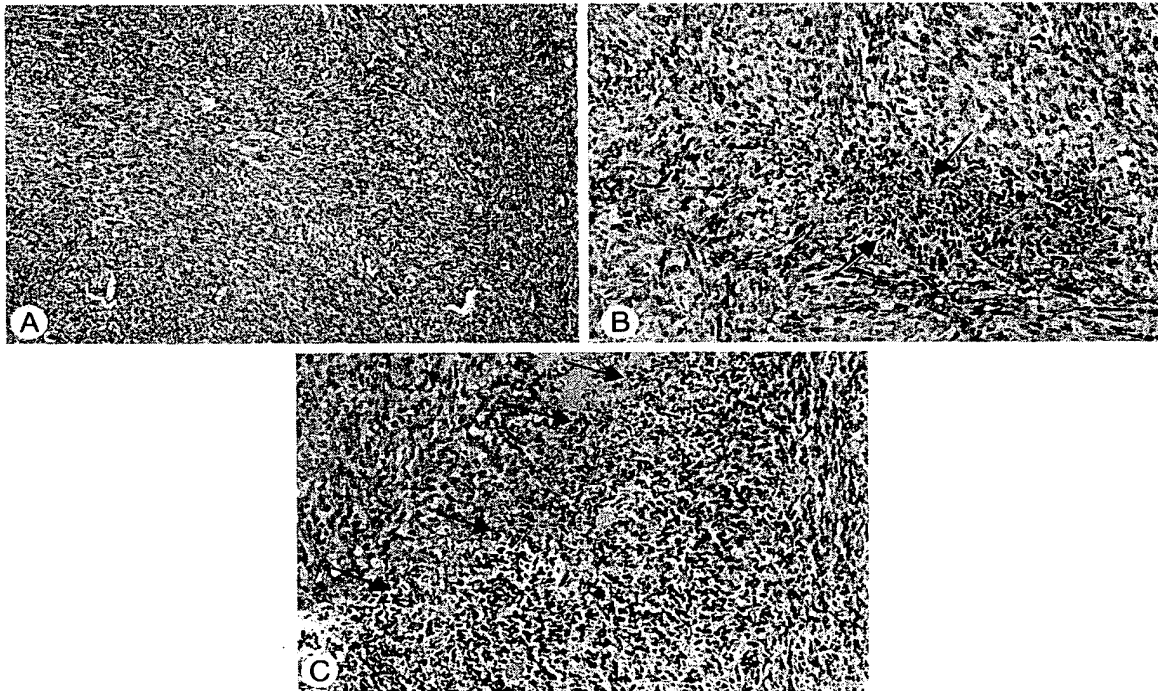


Figure 7. Histological photomicrographs (hematoxylin and eosin) of MDA-MB-231 tumor. **A:** Section from a MDA-MB-231 tumor showing poorly differentiated, spindle-shaped tumor cells with brisk mitotic activity. **B:** Section from a MDA-MB-231 tumor injected with AdV-pLpA showing small foci of necrosis (arrows). **C:** Section from a MDA-MB-231 tumor injected with AdV-p16 showing extensive areas of necrosis (arrows). Magnifications: A, $\times 55$; B, $\times 55$; C, $\times 55$.

express p16^{INK4}. Recently, several studies have also shown that high levels of exogenous p16^{INK4} expression resulted in the delayed appearance of apoptosis.³⁵⁻³⁷

The effect of a sustained G₁ block by p16^{INK4} is probably caused by repression of Rb expression in response to the accumulation of nonphosphorylated Rb and its proteolytic degradation.^{38,39} The apoptosis response to sustained growth arrest may be due to the down-regulation of Rb seen in tumor cells with exogenous expression of p16^{INK4}.⁴⁰ When Rb levels are sufficiently reduced, the protective role of Rb toward apoptosis can no longer be maintained. Our results are thus consistent with a recent report that AdV-p16 infection was cytotoxic to cancer cells that expressed wt Rb but were null or mutant for p16^{INK4}; the study further showed that the infection was much less cytotoxic to cancer cells that did not express a wt Rb but expressed wt p16^{INK4}.⁴¹

In the BALB/c *nu/nu* mouse model, our data showed that i.t. injection of AdV-pLpA alone resulted in some degree of tumor growth inhibition and in the formation of multiple small tumor necrosis. These are possibly derived from the immediate inflammatory immune responses directed against the adenovirus, which consist of the early influx of neutrophils, natural killer cells, and macrophages into infected tissues.⁴² More importantly, our data also showed that (a) the exogenous expression of p16^{INK4} is able to reduce the tumorigenicity of infected tumor cells, and (b) the i.t. injection of AdV-p16 is able to significantly inhibit tumor growth *in vivo* and induce extensive tumor necrosis. Our results thus suggest that adenovirus-mediated p16^{INK4} gene transfer may represent a new therapeutic approach for the treatment of breast cancers.

In summary, this report shows that infection of human breast cancer cells with AdV-p16, a recombinant adenovirus expressing p16^{INK4}, resulted in high levels of p16 expression. This marked increase in expression of the exogenous p16^{INK4} is able to (a) induce cell cycle arrest in the G₁ phase of tumor cells expressing functional endogenous Rb, but not in cells lacking functional Rb, (b) induce apoptotic cell death *in vitro*, and (c) significantly inhibit pre-established breast tumors *in vivo* in an animal model. Hence, the current study suggests that replacement of the wt p16^{INK4} gene may be an effective molecular approach in the treatment of human breast cancers.

ACKNOWLEDGMENTS

This work was supported by a research grant from the Medical Research Council of Canada. I.C. was supported by a Graduate Scholarship from the Saskatchewan Cancer Agency.

REFERENCES

- Weinberg R. Tumor suppressor genes. *Science*. 1991;254:1138-1146.
- Sugimura T, Terada M, Yokota J, Hirohashi S, Wakabayashi K. Multiple genetic alterations in human carcinogenesis. *Environ Health Perspect*. 1992;98:5-12.
- Friedman T. Progress toward human gene therapy. *Science*. 1989;244:1275-1281.
- Friedman T. Gene therapy of cancer through restoration of tumor-suppressor functions. *Cancer*. 1992;70(suppl):1810-1817.
- Noda A, Ning Y, Venable S, Pereira-Smith O, Smith J. Cloning of senescent cell-derived inhibitors of DNA synthesis using an expression screen. *Exp Cell Res*. 1994;211:90-8.
- Serrano M, Hannon G, Beach D. A new regulator motif in cell-cycle control causing specific inhibition of cycling D/CDK4. *Nature*. 1993;366:704-7.
- Cheng J, Jhanwar S, Klein W, et al. P16 alterations and deletion mapping of 9p21-p22 in malignant mesothelioma. *Cancer Res*. 1994;54:5547-5551.
- Kamb A, Gruis N, Weaver-Feldhaus J, et al. A cell cycle regulator potentially involved in genes of many tumor types. *Science*. 1994;264:436-440.
- Nobori T, Miura K, Wu D, Lois A, Takabayashi K, Carson D. Deletion of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. *Nature*. 1994;368:753-756.
- Merla A, Herman J, Mao L, et al. 5' CpG island methylation is associated with transcriptional silencing of the tumor suppressor p16/CDK2/MTS1 in human cancers. *Nat Med*. 1995;1:686-692.
- Ewen M. The cell cycle and the retinoblastoma protein family. *Cancer Metastasis Rev*. 1994;13:45-66.
- Chen P, Riley D, Lee W. The retinoblastoma protein as a fundamental mediator of growth and differentiation signals. *Crit Rev Eukaryot Gene Expr*. 1995;5:79-95.
- Grana X, Reddy E. Cell cycle control in mammalian cells: role of cyclins, cyclin-dependent kinases (CDKs), growth suppressor genes, and cyclin-dependent kinase inhibitors (CKIs). *Oncogene*. 1995;11:211-219.
- Jin X, Nguyen D, Zhang W, Kyritsis A, Roth J. Cell cycle arrest and inhibition of tumor cell proliferation by the p16 gene mediated by a adenovirus vector. *Cancer Res*. 1995;55:3250-3252.
- Schrump D, Chen G, Consoli U, Jin X, Roth J. Inhibition of esophageal cancer proliferation by adenovirally mediated delivery of p16. *Cancer Gene Ther*. 1996;6:357-364.
- Fueyo J, Gomez-Manzano C, Yung W, et al. Adenovirus-mediated p16/CDK2 gene transfer induces growth arrest and modifies the transformed phenotype of glioma cells. *Oncogene*. 1996;12:103-110.
- Feuer E. The lifetime risk of developing breast cancer. *J Natl Cancer Inst*. 1993;85:892-897.
- Boring C. Cancer statistics. *CA Cancer J Clin*. 1994;44:7-26.
- Xu L, Sgroi D, Sterner C, et al. Mutational analysis of CDKN2 (MTS1/p16INK4) in human breast carcinomas. *Cancer Res*. 1994;54:5262-5264.
- Bern E, Klijn J, Smid M, Staveren I, Gruis N, Foekens J. Infrequent CDKN2 (MTS1/p16) gene alterations in human primary breast cancer. *Br J Cancer*. 1995;72:964-967.
- Liu Q, McClure M, Frye C, et al. CDKN2 (MTS1) tumor suppressor gene mutation in human tumor cell lines. *Oncogene*. 1995;10:1060-1067.
- Quesnel B, Fenauz P, Philippe N, et al. Analysis of p16 gene deletion and point mutation in breast carcinoma. *Oncogene*. 1995;10:351-353.



23. Rush E, Abouezzi Z, Borgen P, Anelli A. Analysis of MTS1/CDK4 in female breast carcinomas. *Cancer Lett.* 1995;89:223-226.
24. Geradts J, Wilson P. High frequency of aberrant p16INK4A expression in human breast cancer. *Am J Pathol.* 1996;149:15-20.
25. Brody S, Jaffee H, Han S, Wersto R, Crystal R. Direct in vivo gene transfer and expression in malignant cells using adenovirus vectors. *Hum Gene Ther.* 1994;5:437-447.
26. Chen S, Shine H, Goodman J, Grossman R, Woo A. Gene therapy for brain tumors: regression of experimental gliomas by adenovirus-mediated gene transfer in vivo. *Proc Natl Acad Sci USA.* 1994;91:3054-3057.
27. Gotoh A, Kao C, Ko S, Hamada K, Liu T, Chung L. Cytotoxic effects of recombinant adenovirus p53 and cell cycle regulator genes (p21 and p16) in human prostate cancers. *J Urol.* 1997;158:636-641.
28. Wright P, Zheng C, Moyana T, Xiang J. Intratumoral vaccination of adenovirus expressing fusion protein RM4/TNF induces significant tumor regression. *Cancer Gene Ther.* 1998;5:371-379.
29. Mehta R, Graves J, Hart G, Shilkaitis A, Gupta T. Growth and metastasis of human breast carcinomas with Matrigel in athymic mice. *Breast Cancer Res Treat.* 1993;25:65-71.
30. Herman J, Merlo A, Mao L, et al. Inactivation of the CDKN2/p16/MTS1 gene is frequently associated with aberrant DNA methylation in all common human cancers. *Cancer Res.* 1995;55:4525-4530.
31. Emig R, Magener A, Ehemann V, et al. Aberrant cytoplasmic expression of the p16 protein in breast cancer is associated with accelerated tumor proliferation. *Br J Cancer.* 1998;78:1661-1668.
32. Sano T, Oyama T, Kashiwabara K, Fukuda T, Nakajima T. Immunohistochemical overexpression of p16 protein associated with intact retinoblastoma protein expression in cervical cancer and cervical intraepithelial neoplasia. *Pathol Int.* 1998;48:580-585.
33. Macaulay R, Wang W, Dimitroulakos J, Becker L, Yeger H. Lovastatin-induced apoptosis of human medulloblastoma cell lines in vitro. *J Neurooncol.* 1999;42:1-11.
34. Serrano M, Hannon G, Beach D. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature.* 1993;366:704-707.
35. Schreiber M, Muller W, Singh G, Graham F. Comparison of the effectiveness of adenovirus vectors expressing cyclin kinase inhibitors p16^{INK4A}, p18^{INK4C}, p19^{INK4D}, p21^{WAF1/CIP1}, and p27^{KIP1} in inducing cell cycle arrest, apoptosis, and inhibition of tumorigenicity. *Oncogene.* 1998;16:1663-1676.
36. Frizelle S, Grim J, Zhou J, Gupta P, Curiel D, Geradts J. Re-expression of p16INK4A in mesothelioma cells results in cell cycle arrest, cell death, tumor suppression and tumor regression. *Oncogene.* 1998;16:3087-3095.
37. Naruse I, Heike Y, Hama S, Mori M, Saijo N. High concentrations of recombinant adenovirus expressing p16 gene induce apoptosis in lung cancer cell lines. *Anticancer Res.* 1998;18:4275-4282.
38. Gill R, Hamel PA, Zhe J, Zacksenhaus E, Gallie BL, Phillips RA. Characterization of the human Rb promoter and of elements involved in transcriptional regulation. *Cell Growth Differ.* 1994;5:467-474.
39. An B, Dou Q. Cleavage of retinoblastoma protein during apoptosis: an IL-1 β -converting enzyme-like protease as a candidate. *Cancer Res.* 1996;56:438-442.
40. Sandig V, Brand K, Herwig S, Lukas J, Bartek J, Strauss M. Adenovirally transferred p16INK4/CDKN2 and p53 genes cooperate to induce apoptotic tumor death. *Nat Med.* 1997;3:313-319.
41. Craig C, Kim M, Ohri E, et al. Effects of adenovirus-mediated p16 expression on cell cycle arrest are determined by endogenous p16 and Rb status in human cancer cells. *Oncogene.* 1997;16:265-272.
42. Adesanya M, Redman R, Baum B, O'Connell B. Immediate inflammatory responses to adenovirus-mediated gene transfer in rat salivary glands. *Hum Gene Ther.* 1996;7:1085-1093.



ACQUIRED DISEASES

RESEARCH ARTICLE

Direct intra-cardiomuscular transfer of β_2 -adrenergic receptor gene augments cardiac output in cardiomyopathic hamsters

K Tomiyasu¹, Y Oda¹, M Nomura², E Satoh², S Fushiki³, J Imanishi², M Kondo¹ and O Mazda²

¹First Department of Internal Medicine and ²Department of Microbiology and ³Department of Dynamic Pathology, Research Institute for Neurological Disease and Geriatrics, Kyoto Prefectural University of Medicine, Kamikyo, Kyoto, Japan

In chronic heart failure, down-regulation of β -adrenergic receptor (β -AR) occurs in cardiomyocytes, resulting in low catecholamine response and impaired cardiac function. To correct the irregularity in the β -AR system, β -AR gene was transduced *in vivo* into failing cardiomyocytes. The Epstein–Barr virus (EBV)-based plasmid vector carrying human β_2 -AR gene was injected into the left ventricular muscle of Bio14.6 cardiomyopathic hamsters whose β -AR is down-regulated in the cardiomyocytes. The echocardiographic examinations revealed that stroke volume (SV) and cardiac output (CO) were significantly elevated at 2 to 4 days after

the β_2 -AR gene transfer. Systemic loading of isoproterenol increased the cardiac parameters more significantly on day 2 to day 7, indicating that the adrenergic response was augmented by the genetic transduction. The same procedure did not affect the cardiac function of normal hamsters. Immunohistochemical examinations demonstrated human β_2 -AR expression in failing cardiomyocytes transduced with the gene. RT-PCR analysis detected mRNA for the transgene in the heart but not in the liver, spleen, or kidney. The procedures may provide a feasible strategy for gene therapy of severe heart failure. Gene Therapy (2000) 7, 2087–2093.

Keywords: heart failure; β -adrenergic receptor; Epstein–Barr virus-based-plasmid vector; cardiomyopathic hamster; gene therapy

Introduction

In chronic cardiac failure, persistent excitation of the sympathetic nervous system ultimately leads to down-regulation of the β -AR in cardiac cells, contributing to the decrease in cardiac function. If a large number of β -AR can be expressed in cardiomyocytes through genetic transduction technique, reactivity to catecholamines may be improved, enabling the possible recovery of cardiac contractility.^{1–3} Milano *et al*⁴ established a transgenic mouse constitutively expressing β_2 -AR in the heart. They found a significant increase in the heart rate (HR), as well as improvement in the dp/dt max, an index of isochronic contractility. More recently, Kawahira *et al*⁵ and Kypson *et al*⁶ transduced the β_2 -AR gene *ex vivo* into donor hearts isolated from pressure-overloaded and normal rats, respectively. They found elevated LV dp/dt max in the heart graft heterotopically transplanted into recipient animals. Maurice *et al*⁷ transduced a normal rabbit heart *in vivo* with the β_2 -AR gene resulting in the elevation of LV dp/dt max. Although these earlier reports strongly suggest the feasibility of the β_2 -AR gene transfer in terms of gene therapy for heart failure, there have been no reports showing *in vivo* transduction of the β_2 -AR gene into failing hearts, with attendant cardiac functional restoration.

To express a large number of β_2 -AR in failing cardiac muscle cells, it is necessary to find ways to conduct genetic transduction as efficiently as possible. Previously, we reported that a high rate of gene transduction could be achieved in cardiomyocytes *in vivo* through injection of the Epstein–Barr virus (EBV)-based plasmid.⁸ In the present study, we used this method to transduce the β_2 -AR gene into the hearts of cardiomyopathic hamsters. Because direct injection of naked plasmid DNA allows transgene expression specifically in cardiac muscle, and employment of the EBV-based plasmids enables highly efficient gene transduction, our system is considered to be highly appropriate for gene therapy of heart failure.

Results

We first estimated the transduction efficiency *in vivo* into cardiac muscle using our system. pSES. β (Figure 1) was inoculated into the hearts of the F1b hamsters, and X-gal staining was performed. The β -gal expression was demonstrated in cardiac muscle within approximately 0.1 cm from each injection site (Figure 2a). This is compatible with our previous finding using normal rat heart.⁸

Next, the Bio14.6 hamsters were injected with pSES. β_2 -AR or pSES. β into the left ventricles, and their cardiac function was evaluated before and 2 and 4 days after the transduction. Figure 3 and Table 1 summarize the cardiac function parameters obtained by the echocardiographic analyses. The hamsters transduced with pSES. β_2 -AR gene showed 1.6-fold higher cardiac output (CO) than the

Correspondence: O Mazda, Department of Microbiology, Kyoto Prefectural University of Medicine, Kamikyo, Kyoto 602-8566, Japan
Received 11 April 2000; accepted 30 August 2000

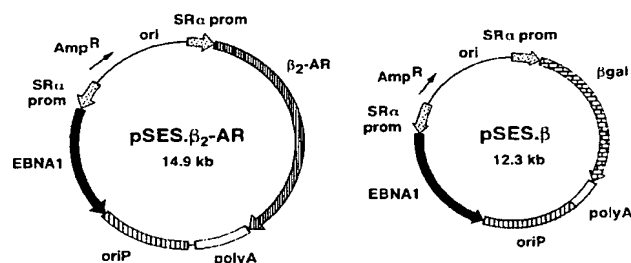


Figure 1 Plasmids used in this study. Maps of pSES.β₂-AR (left) and pSES.β (right)^{6,7} are shown. Prom, promoter; polyA, SV40 polyA additional signal.

pSES.β-transduced animals on day 4 after the gene transfer (45.6 ± 21.0 ml versus 70.8 ± 21.3 ml, $P < 0.05$) (Figure 3, left lower panel). Cardiac response to isoproterenol, a β-AR agonist, was also studied by systemic loading of the drug. On day 4, pSES.β₂-AR-transduced group showed 1.5-, 1.3- and 1.5-fold higher value for fractional shortening (FS), stroke volume (SV) and CO, respectively, in comparison with the pSES.β-transduced group (FS: 22.7 ± 1.5 ml versus 14.9 ± 5.7 ml, $P < 0.05$; SV: 0.256 ± 0.048 ml versus 0.204 ± 0.081 ml, $P < 0.05$; CO: 95.8 ± 20.7 ml versus 63.5 ± 31.8 ml, $P < 0.05$) (Figure 3, right panels). In the group given pSES.β₂-AR, the FS was 1.3-fold higher than that obtained before transduction. (FS: 22.7 ± 1.5 ml versus 17.0 ± 2.9 ml, $P < 0.05$) (Figure 3, right upper panel). The SV and CO of the gene-transduced animals were elevated 1.7- and 1.8-fold on day 2, respectively, in comparison with the levels before the genetic transduction (SV: 0.296 ± 0.023 ml versus 0.170 ± 0.037 ml, $P < 0.005$; CO: 112.3 ± 16.7 ml versus 62.9 ± 13.3 ml, $P < 0.005$) (Figure 3, right middle and lower panels). The results indicate that intramyocardial injection of pSES.β₂-AR elicits improvement in cardiac function, as well as augmentation of adrenergic response in the cardiomyopathic hamsters.

To examine the influence of gene transduction on cardiac function of normal animals, the F1b hamsters were also tested. Compared with Bio14.6, the F1b hamsters show smaller SV and CO, due to smaller left ventricular internal dimension at end-diastole (Dd) and left ventricular internal dimension at end-systole (Ds) (Tables 1 and 2). When the F1b were transduced with pSES.β₂-AR or pSES.β, no significant change was observed in the parameters before and subsequent to genetic transduction, regardless of the plasmid injected (Table 2). Systemic administration with isoproterenol showed that the adrenergic response in pSES.β₂-AR-transduced F1b was comparable to that in F1b given transduction with control plasmid.

Next, the parameters for cardiac function were computed for the Bio14.6 animals that received pSES.β₂-AR or pSES.β transduction, and kinetic changes were monitored up to the 9th day following transduction. In the group transduced with pSES.β₂-AR, significant elevation in the SV and CO was seen on day 2 to 7, compared with the levels before genetic transduction (Figure 4).

The human β₂-AR was demonstrated in cardiomyocytes from the pSES.β₂-AR-transduced Bio14.6 by immunohistochemical staining (Figure 2b). In contrast, the hearts of pSES.β-transduced Bio14.6 did not stain

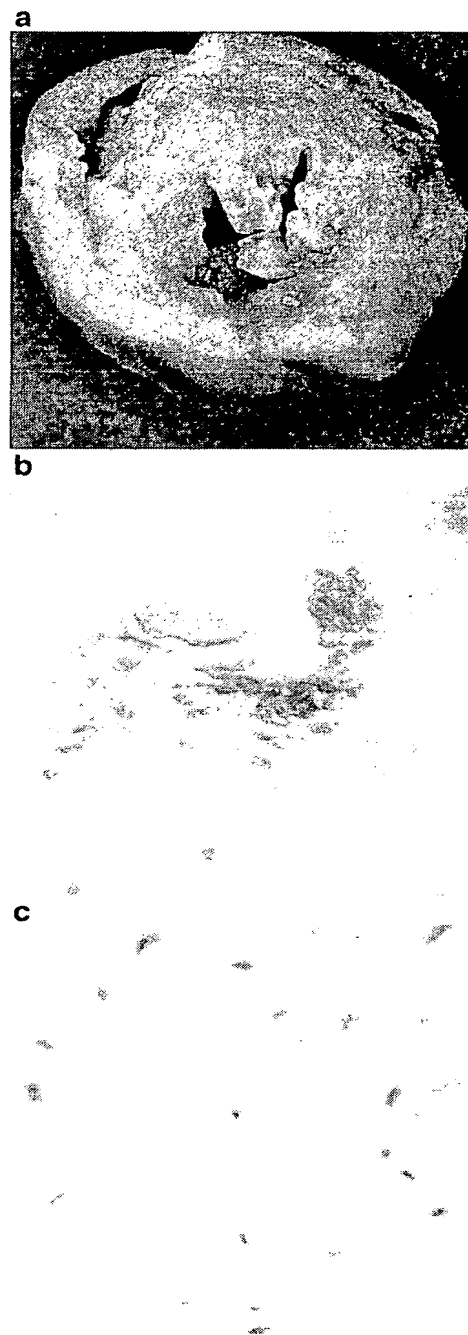


Figure 2 Gene transfer in vivo by intra-myocardial injection of plasmid DNA. (a) Three hundred μg of pSES.β was injected into the left ventricle of F1b, and 5 days later, the animal was killed. The heart was fixed and stained with X-gal. (b and c) Three hundred μg of pSES.β₂-AR (b) or pSES.β (c) was injected into the left ventricle of the Bio14.6, and 5 days later, the animal was killed. A frozen section of the heart was immunostained with anti-human β₂-AR antibody.

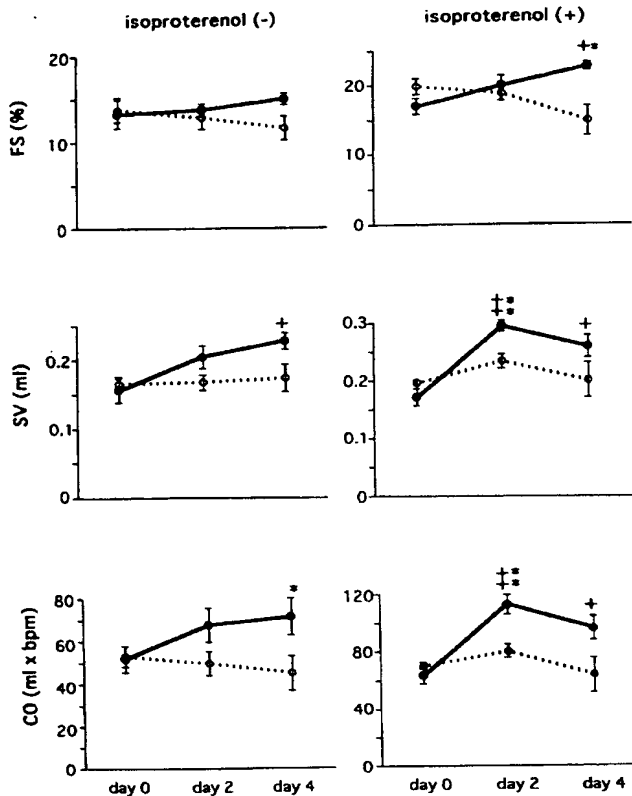


Figure 3 Improvement of cardiac function of gene-transduced Bio14.6 cardiomyopathic hamsters. The Bio14.6 were transduced with pSES.β₂-AR (solid lines, n = 7) or pSES.β (dotted lines, n = 6). On the indicated days after the transduction, echocardiographic examination was performed before (left panels) or 5 min after (right panels) percutaneous administration of 0.1 mg/kg of isoproterenol. Results are expressed as mean ± s.d. (+: P < 0.05, ++: P < 0.005 in comparison with the parameters for pre-transduction; *: P < 0.05, **: P < 0.005 in comparison between pSES.β₂-AR-transduced and pSES.β-transduced groups).

with the anti-human β₂-AR antibody that does not recognize hamster β₂-AR (Figure 2c). RT-PCR experiments demonstrated the transgene-specific mRNA in pSES.β₂-AR-injected heart, but not in liver, kidney or spleen of the same animal (Figure 5). The sense and antisense PCR primers were set corresponding to SRα and β₂-AR sequences, respectively, so that endogenous β₂-AR mRNA is not amplified. The results indicate that the transgene expression was achieved exclusively in the heart by intra-myocardial injection of the naked EBV-based plasmid vector.

Discussion

In the present study, we succeeded in augmenting cardiac function in cardiomyopathic hamsters by transfecting β₂-AR gene into the cardiac muscle, in an effort to correct irregularities in the β-AR system of failing cardiomyocytes. Administration with isoproterenol resulted in an even more significant improvement in cardiac function. The increase in CO may be ascribed to an increase in the SV rather than that in the HR. As far as we know,

there have been no reports showing *in vivo* genetic transduction into failing cardiomyocytes.

Unlike the β₂-AR transgenic mouse, the β₁-AR transgenic mouse did not show elevated cardiac contractility.^{9,10} Therefore, the β₂-AR gene may be more suitable for gene therapy of heart failure than the β₁-AR gene.

The *in vivo* genetic transduction into heart tissue has been done using many different methods, including direct intra-myocardial injection of naked plasmid DNA¹¹ and adenoviral vector,¹² and intra-coronary infusion of adenoviral,¹³ HVJ-liposome¹⁴ and AAV¹⁵ vectors. We chose the direct intra-muscular injection method so that the gene expression is limited exclusively to cardiac muscle. Actually, we did not detect any evidence for transgene expression in organs other than the heart by RT-PCR (Figure 5). Another advantage of this method is simplicity and safety. Unlike healthy animals, the physiological data of emaciated animals like cardiomyopathic hamsters fluctuate considerably with even a small degree of invasion, and for this reason, it is necessary to minimize the amount of invasion as much as possible. Provided that direct injection is carried out under echocardiography guidance, *in vivo* naked plasmid injection can be done percutaneously without thoracotomy, so that the degree of invasion with this method is very low. On the other hand, direct injection to cardiac muscle is advantageous over the intra-coronary approach in that the former is also considered appropriate for genetic transduction to cells in an ischemic lesion barely supplied with blood flow. When cardiac incompetence caused by ischemia is considered as a target disease for gene therapy, this point may become important.

An impediment to naked plasmid injection is that the intensity of gene expression is comparatively low. To overcome this problem, we used the EBV-based plasmid vector, which carries oriP and EBNA1 gene from EBV genome. The EBNA1-oriP system facilitates nuclear localization of the plasmid, anchorage of plasmid to nuclear matrix, and transcriptional up-regulation. Previously, we have reported the effectiveness of the vectors in transfecting various cells of human origin both *in vitro* and *in vivo*.^{8,16–20} We also reported that by using the EBV-based plasmid vectors, *in vitro* transient expression over 10 times higher than that by using conventional plasmids was observed, not only in primate cells but in various types of rodent cells as well.⁸ Furthermore, by directly injecting the EBV-plasmid into rat heart, higher rates of genetic transduction and expression were obtained than by injecting conventional plasmids.⁸

With naked plasmid injection, genetic transduction is only possible in a limited portion of the heart. We cautiously injected a small amount of a DNA solution into five locations of the heart, i.e. the front, rear, and side walls of the left ventricle, the septum, and the apex of the heart, to achieve gene expression over a comparatively wide range of cardiac muscle (Figure 2a). On the other hand, cardiomyocytes are connected by the gap junctions, so that adjacent cells are biochemically aligned and function cooperatively. It has been reported that cAMP, the second messenger mediating adrenergic stimulation, can be transmitted through gap junctions.^{21–23} Taking advantage of this cell-to-cell communication, it may be possible to increase the contractility of cardiac cells which fail to be transduced, but which are connected to neighboring

Table 1 Cardiac function parameters of gene-transduced animals under isoproterenol(-)

Hamster transfection	Bio 14.6						F1b					
	pSES- β (n=6)			pSES- β_2 AR (n=7)			pSES- β (n=5)			pSES- β_2 AR (n=4)		
Days	0	2	4	0	2	4	0	2	4	0	2	4
HR, bpm	317 ± 38	290 ± 56	246 ± 73	329 ± 20	325 ± 39	308 ± 64	370 ± 44	323 ± 43	368 ± 56	407 ± 14	334 ± 41	396 ± 46
FS, %	13.7 ± 3.8	12.7 ± 3.4	11.4 ± 3.6	13.2 ± 4.1	13.7 ± 1.9	14.8 ± 1.7	45.8 ± 4.7	37.6 ± 6.2	39.0 ± 2.9	34.5 ± 6.4	39.8 ± 7.5	37.0 ± 2.3
Dd, mm	7.8 ± 0.4	8.0 ± 0.5	8.2 ± 0.4	7.7 ± 0.7	8.2 ± 0.5	8.2 ± 0.3	4.5 ± 0.2	5.0 ± 0.3	5.2 ± 0.6	4.9 ± 0.5	5.1 ± 0.7	4.9 ± 0.2
Ds, mm	6.7 ± 0.6	7.0 ± 0.7	7.2 ± 0.4	6.7 ± 0.7	7.0 ± 0.4	6.9 ± 0.4	2.5 ± 0.3	3.2 ± 0.4	3.2 ± 0.3	3.1 ± 0.4	3.1 ± 0.7	3.1 ± 0.1
SV, ml	0.167 ± 0.028	0.171 ± 0.029	0.174 ± 0.053	0.156 ± 0.041	0.203 ± 0.04	0.228 ± 0.029	0.077 ± 0.010	0.095 ± 0.021	0.106 ± 0.036	0.087 ± 0.027	0.106 ± 0.036	0.087 ± 0.015
CO, ml × bpm	53.0 ± 12.9	49.1 ± 14.8	45.6 ± 21.0	51.6 ± 15	67.2 ± 19.3	70.8 ± 21.3	28.7 ± 3.2	31.0 ± 8.2	45.5 ± 13.0	35.5 ± 10.5	35.6 ± 12.8	34.1 ± 1.5

Values are mean ± s.d.

Table 2 Cardiac function parameters of gene-transduced animals under isoproterenol(+)

Hamster transfection	Bio 14.6						F1b					
	pSES- β (n=6)			pSES- β_2 AR (n=7)			pSES- β (n=5)			pSES- β_2 AR (n=4)		
Days	0	2	4	0	2	4	0	2	4	0	2	4
HR, bpm	358 ± 29	345 ± 47	306 ± 66	370 ± 16	382 ± 45	372 ± 49	420 ± 17	389 ± 20	424 ± 31	448 ± 12	389 ± 40	404 ± 40
FS, %	19.9 ± 3.2	18.9 ± 2.8	14.9 ± 5.7	17 ± 2.9	20 ± 3.3	22.7 ± 1.5	53.2 ± 3.6	54.8 ± 6.4	55.4 ± 5.3	53.3 ± 4.5	55.5 ± 3.4	60.2 ± 3.7
Dd, mm	7.9 ± 0.3	7.9 ± 0.4	8.1 ± 0.5	7.4 ± 0.6	8.3 ± 0.3	7.8 ± 0.5	4.2 ± 0.3	4.3 ± 0.3	4.1 ± 0.5	4.6 ± 0.5	4.4 ± 0.5	4.4 ± 0.2
Ds, mm	6.0 ± 0.5	6.4 ± 0.5	6.9 ± 0.7	6.2 ± 0.6	6.5 ± 0.5	6.1 ± 0.4	2.0 ± 0.3	1.9 ± 0.3	1.8 ± 0.3	2.2 ± 0.4	2.0 ± 0.3	2.1 ± 0.6
SV, ml	0.197 ± 0.014	0.233 ± 0.033	0.204 ± 0.081	0.17 ± 0.037	0.296 ± 0.023	0.256 ± 0.048	0.067 ± 0.011	0.071 ± 0.014	0.063 ± 0.274	0.087 ± 0.029	0.078 ± 0.027	0.074 ± 0.018
CO, ml × bpm	70.5 ± 6.9	79.9 ± 12.9	63.5 ± 31.8	62.9 ± 13.3	112.3 ± 16.7	95.8 ± 20.7	27.9 ± 5.3	28.0 ± 6.1	27.7 ± 11.7	39.2 ± 13.3	30.4 ± 11.4	29.5 ± 5.0

Values are mean ± s.d.

cells that are successfully transduced with the β_2 -AR gene.

In our experiments, no significant change was observed in cardiac function of normal hamsters, while Kypson *et al*⁶ and Maurice *et al*⁷ reported significant elevation in LV-dp/dt max in normal animals given adenovirus vector-mediated transduction of β_2 -AR gene. Because our method involved plasmid injection to cardiac muscle, the intensity of β -AR expression may be lower than that in their experiments. Alternatively, the discrepancy may be ascribed to the different parameters measured as an index of cardiac function. We analyzed cardiac function of animals less invasively and more physiologically using echocardiography,²⁴ while both Kypson *et al*,⁶ Maurice *et al*⁷ and Kawahira *et al*⁵ measured LV-dp/dt max by means of cardiac catheterization.

A variety of therapeutic approaches have been devised to augment the β -adrenergic signaling to compensate cardiac function in heart failure (eg administration with dobutamine,²⁵ prenalterol,²⁶ xamoterol,²⁷ and milrinone²⁸). These therapeutic strategies actually improve clinical symptoms during short periods following administration, but fail to significantly prolong the

survival of patients. This may be ascribed to β -AR down-regulation in failing cardiomyocytes. Therefore, β -AR gene transfer may be advantageous over pharmacologic interventions. In contrast, β -AR blockade was revealed to be potentially useful for long-term treatment.^{29,30} The concepts are compatible with the manifestations of the transgenic animals overexpressing the myocardial Gs α , a component of β -AR signaling pathway.³¹ In cases of chronic severe heart failure beyond conventional medical treatment, temporary recovery of cardiac function is mandatory. Enhancing cardiac function by a β -AR increase is potentially the treatment of choice. β -AR gene therapy may be advantageous in terms of improvement of quality of life in acute crisis of chronic heart failure, under enhanced sympathetic activation. Short-term adrenergic stimulation may be effective in weaning from large-dose catecholamine therapy, as well as the introduction of β -AR blockade therapy in severe cases. The therapy may also be applicable to temporal restoration of severe heart failure in patients to be treated by heart transplantation.

In conclusion, improved cardiac function was observed as a result of *in vivo* β_2 -AR genetic transduction in an animal model with failing heart. This suggests new possibilities in molecular treatment of severe cardiac failure in which there are obstacles to the β -AR system, and which are resistant to conventional medical treatment.

Materials and methods

Plasmids

pSES. β_2 -AR and pSES. β are plasmid vectors containing the expression units for the human β_2 -AR cDNA (*EcoRI*-

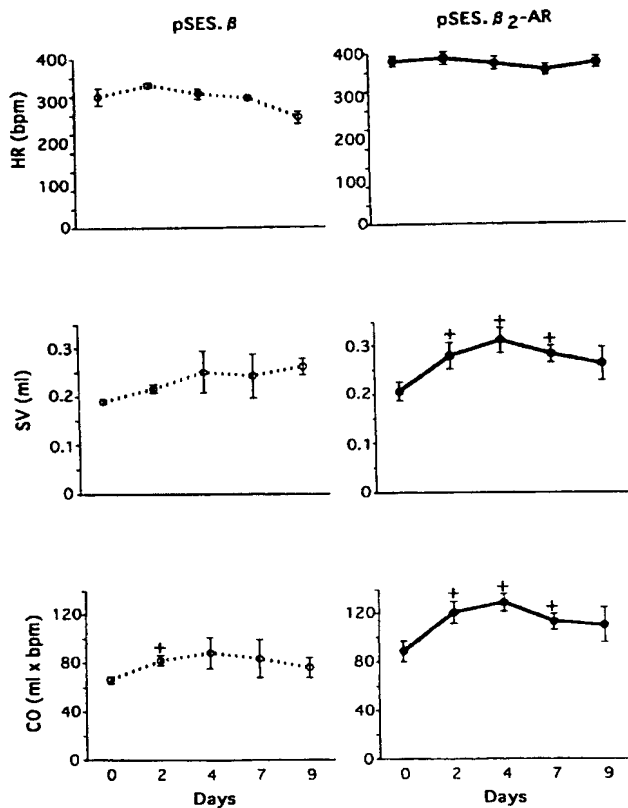


Figure 4 Kinetic change in cardiac function of gene-transduced Bio14.6 cardiomyopathic hamsters. The Bio14.6 were transduced with pSES. β_2 -AR (right panels, $n = 7$) or pSES. β (left panels, $n = 3$). On the indicated days after the transduction, echocardiographic examination was performed 5 min after percutaneous administration of 0.1 mg/kg of isoproterenol. Results are expressed as mean \pm s.d. (+: $P < 0.05$ in comparison with pre-transduction.)

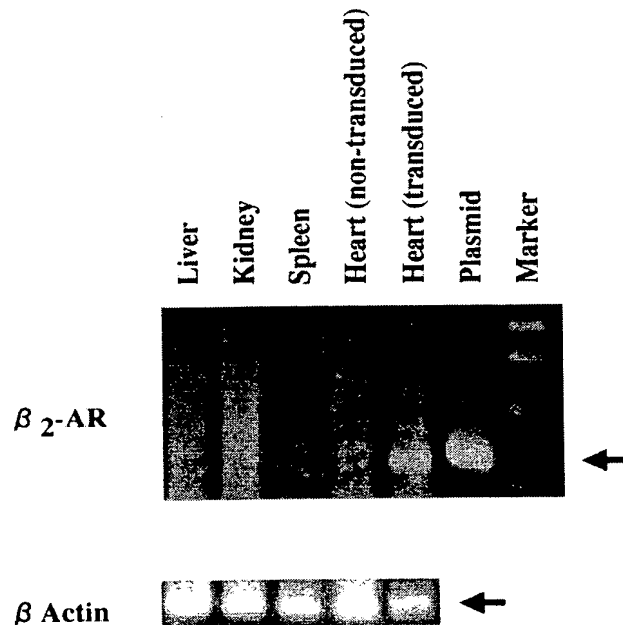


Figure 5 Transgene is exclusively expressed in the heart. The Bio14.6 were transduced with pSES. β_2 -AR, and 10 days later, RNA was extracted from the indicated organs. RT-PCR was performed using the combinations of SR α -specific sense and human β_2 -AR gene-specific antisense primers (upper panel) or β -actin gene-specific sense and antisense primers (lower panel).

Sall 1.8 kb fragment from pTZ β_2 -AR kindly provided by Dr M Bouvier³²⁻³⁴ and *E. coli* β -gal gene, respectively. Both plasmids also possess the EBV oriP element, as well as the EBV nuclear antigen 1 (EBNA1) gene (Figure 1).

Animals

Bio14.6 (cardiomyopathic hamsters) and F1b (normal golden hamsters) were purchased from Charles River Laboratories (Wilmington, MA, USA). As Bio14.6 grow older, they show manifestations of heart failure, β -ARs being down-regulated in the cardiomyocytes.³⁵ The FS of the Bio14.6 were 46.8 ± 4.17 and 13.46 ± 3.78 at 29 and 45 weeks of age, respectively, while that of the 45-week-old F1b was 40.78 ± 7.86 . Therefore, we used 45-week-old Bio14.6 for the experiments.

Gene transfer

The animals were anesthetized by intra-peritoneal injection with sodium thiobarbital (0.1 mg/kg). A DNA/20% sucrose solution (2 μ g/ μ l) containing a small amount of carbon particles as a marker was percutaneously injected into the left ventricular wall, using a microsyringe with a 27G needle, under guidance of the echocardiography (Digital Sonolayer PowerVision SSA-380A equipped with a 7.5-MHz transducer; Toshiba, Tokyo, Japan). Approximately 40 μ l of the solution was injected at each of five locations.

Measurement of cardiac function

The animals were anesthetized by intra-peritoneal injection with sodium thiobarbital (0.1 mg/kg) and analyzed by blinded observers. The HR, FS, Dd and Ds parameters were individually measured by the echocardiographic device before or 5 min after percutaneous administration of 0.1 mg/kg of isoproterenol. The SV and CO were calculated according to following formula:

$$SV = Dd^3 - Ds^3 \\ CO = SV \times HR$$

Statistical analysis

Differences in cardiac function data were assessed by use of a two way repeated-measures ANOVA. If a significant F ratio was obtained, further analysis was carried out with Fisher's PLSD *post hoc* test. Differences between groups were considered significant at $P < 0.05$.

X-gal staining

The F1b were killed on day 5 following pSES. β transduction. The hearts were excised, cut into slices and fixed with 2% paraformaldehyde for 4 h at 4°C. Following thorough rinsing with PBS, the slices were incubated in an X-gal staining solution (0.05% (v/v) 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal) (Nacalai Tesque, Kyoto, Japan), 1 mM MgCl₂, 150 mM NaCl, 3 mM K₄[Fe(CN)₆], 3 mM K₃[Fe(CN)₆], 60 mM Na₂HPO₄, 40 mM NaH₂PO₄ and 0.1% Triton X-100) for 3 h at 37°C.

β_2 -AR immunohistochemistry

The Bio14.6 were killed on day 5 after pSES. β_2 -AR transduction. The hearts were excised, embedded in paraffin, and sliced to a thickness of 5 μ m. The slices were then incubated with an appropriate dilution of anti-human β_2 -AR antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by rinsing with PBS and further incu-

bation with peroxidase-labeled goat anti-rabbit immunoglobulin (EnVision; Dako, Carpinteria, CA, USA). The β_2 -AR expression was visualized by treatment with DAB solution (Dako).

RT-PCR

The organs were removed from the dead animals and rinsed with PBS. RNA was extracted with ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol, and RT-PCR was performed as described elsewhere.³⁶ The combinations of SR α -specific sense and β_2 -AR gene-specific antisense primers (5'-CCGCC TGTGGTGCCTCCTGA and 5'-TGCCCATGCCACCA CCCAC, respectively) or β -actin gene-specific sense and antisense primers (5'-GTGCTATCCCTGTACGCCTC and 5'-AGTCCGCCTAGAAGCATTTG, respectively) were used (Figure 1).

Acknowledgements

We thank Dr M Bouvier for providing the β_2 -AR cDNA. This work was supported by a grant-in-aid for scientific research from the Ministry of Education, Science and Culture, a grant from the Japan Heart Foundation and IBM Japan Research Grant, and a grant from Japan Foundation of Cardiovascular Research.

References

- Kobilka BK *et al.* cDNA for the human β_2 -adrenergic receptor: a protein with multiple membrane-spanning domains and encoded by a gene whose chromosomal location is shared with that of the receptor for platelet-derived growth factor. *Proc Natl Acad Sci USA* 1987; **84**: 46-50.
- Drazner MH *et al.* Potentiation of β -adrenergic signaling by adenoviral-mediated gene transfer in adult rabbit ventricular myocytes. *J Clin Invest* 1997; **99**: 288-296.
- Akhter SA *et al.* Restoration of β -adrenergic signaling in failing cardiac ventricular myocytes via adenoviral-mediated gene transfer. *Proc Natl Acad Sci USA* 1997; **94**: 12100-12105.
- Milano CA *et al.* Enhanced myocardial function in transgenic mice overexpressing the β_2 -adrenergic receptor. *Science* 1994; **264**: 582-586.
- Kawahira Y *et al.* *In vivo* transfer of a β -adrenergic receptor gene into the pressure-overloaded rat heart enhances cardiac response to β -adrenergic agonist. *Circulation* 1998; **98**: II-262-268.
- Kypson AP *et al.* Adenovirus-mediated gene transfer of the β_2 -adrenergic receptor to donor hearts enhances cardiac function. *Gene Therapy* 1999; **6**: 1298-1304.
- Maurice JP *et al.* Enhancement of cardiac function after adenoviral-mediated *in vivo* intracoronary β_2 -adrenergic receptor gene delivery. *J Clin Invest* 1999; **104**: 21-29.
- Tomiyasu K *et al.* Gene transfer *in vitro* and *in vivo* with Epstein-Barr virus-based episomal vector results in markedly high transient expression in rodent cells. *Biochem Biophys Res Commun* 1998; **253**: 733-738.
- Bertin B *et al.* Specific atrial overexpression of G protein coupled human β_1 adrenoceptors in transgenic mice. *Cardiovasc Res* 1993; **27**: 1606-1612.
- Bisognano JD *et al.* Myocardial-directed overexpression of the human β_1 -adrenergic receptor in transgenic mice. *J Mol Cell Cardiol* 2000; **32**: 817-830.
- Lin H *et al.* Expression of recombinant genes in myocardium *in vivo* after direct injection of DNA. *Circulation* 1990; **82**: 2217-2221.
- Stratford-Pericaudet LD, Makeh I, Perricaudet M, Briand P. Widespread long-term gene transfer to mouse skeletal muscles and heart. *J Clin Invest* 1992; **90**: 626-630.

- 13 Barr E *et al.* Efficient catheter-mediated gene transfer into the heart using replication-defective adenovirus. *Gene Therapy* 1994; 1: 51–58.
- 14 Sawa Y *et al.* Efficient gene transfer method into whole heart through the coronary artery with hemagglutinating virus of Japan liposome. *J Thorac Cardiovasc Surg* 1997; 113: 512–519.
- 15 Svensson EC *et al.* Efficient and stable transduction of cardiomyocytes after intramyocardial injection or intracoronary perfusion with recombinant adeno-associated virus vectors. *Circulation* 1999; 99: 201–205.
- 16 Mazda O, Satoh E, Yasutomi K, Imanishi J. Extremely efficient gene transfection by Epstein-Barr virus vectors into lymphohematopoietic cell lines. *J Immunol Meth* 1997; 204: 143–151.
- 17 Satoh E *et al.* Efficient gene transduction by Epstein-Barr virus-based vectors coupled with cationic liposome and HVJ-liposome. *Biochem Biophys Res Commun* 1997; 238: 795–799.
- 18 Satoh E *et al.* Successful transfer of adenosine deaminase (ADA) gene *in vitro* into human peripheral blood CD34⁺ cells by transfecting Epstein-Barr virus (EBV)-based episomal vectors. *FEBS Lett* 1998; 441: 39–42.
- 19 Harada Y *et al.* Highly efficient suicide gene expression in hepatocellular carcinoma cells by Epstein-Barr virus-based plasmid vectors combined with polyamidoamine dendrimer. *Cancer Gene Ther* 2000; 7: 27–36.
- 20 Maruyama-Tabata H *et al.* Effective suicide gene therapy *in vivo* by EBV-based plasmid vectors coupled with polyamidoamine dendrimer. *Gene Therapy* 2000; 7: 53–60.
- 21 Dermot MF, Cooper F, Mons N, Karpen JW. Adenylyl cyclases and the interaction between calcium and cAMP signaling. *Nature (Lond)* 1995; 374: 421–424.
- 22 De Mello WC. Impaired regulation of cell communication by β -adrenergic receptor activation in the failing heart. *Hypertension* 1996; 27: 265–268.
- 23 Kam Y, Kim DY, Koo SK, Joe CO. Transfer of second messengers through gap junction connexin 43 channels reconstituted in liposomes. *Biochim Biophys Acta* 1998; 1372: 384–388.
- 24 Tanaka N *et al.* Transthoracic echocardiography in models of cardiac disease in the mouse. *Circulation* 1996; 94: 1109–1117.
- 25 Krell MJ *et al.* Intermittent, ambulatory dobutamine infusions in patients with severe congestive heart failure. *Am Heart J* 1986; 112: 787–791.
- 26 Lambertz H, Meyer J, Erbel R. Long-term hemodynamic effects of prenalatorol in patients with severe congestive heart failure. *Circulation* 1984; 69: 298–305.
- 27 Xamoterol in Severe Heart Failure Study Group. Xamoterol in severe heart failure. *Lancet* 1990; 336: 1–6.
- 28 Packer M *et al.* Effect of oral milirinone on mortality in severe heart failure. *New Engl J Med* 1991; 325: 1468–1475.
- 29 Heilbrunn SN *et al.* Increased β -receptor density and improved hemodynamic response to catecholamine stimulation during long-term metoprolol therapy in heart failure from dilated cardiomyopathy. *Circulation* 1989; 79: 483–490.
- 30 Waagdsten F *et al.* Long-term β -blockade in dilated cardiomyopathy. *Circulation* 1989; 80: 551–563.
- 31 Iwase M *et al.* Adverse effects of chronic endogenous sympathetic drive induced by cardiac Gs α overexpression. *Circ Res* 1996; 78: 517–524.
- 32 Bouvier M *et al.* Expression of a human cDNA encoding the β_2 -adrenergic receptor in Chinese hamster fibroblasts (CHW): functionality and regulation of the expressed receptors. *Mol Pharmacol* 1987; 33: 133–139.
- 33 Suzuki T *et al.* Distinct regulation of β_1 - and β_2 -adrenergic receptors in Chinese hamster fibroblasts. *Mol Pharmacol* 1991; 41: 542–548.
- 34 Rousseau G, Manter F, Bouvier M. Distinct receptor domains determine subtype-specific coupling and desensitization phenotypes for human β_1 - and β_2 -adrenergic receptors. *Mol Pharmacol* 1996; 49: 752–760.
- 35 Kagiya T, Hori M, Iwai K. Alterations in cardiac [H³] nitrendipine binding in pathogenesis in cardiomyopathic Syrian hamsters (Bio 14.6). *Jpn Circ J* 1986; 50: 664.
- 36 Hirai H *et al.* Use of Epstein-Barr virus-based vector/hemagglutinating virus of Japan-liposome complex vector for targeted gene therapy of Epstein-Barr virus-associated neoplasms. *Biochem Biophys Res Commun* 1997; 241: 112–118.

Int J Gynecol Cancer. 2001 Jan-Feb;11(1):18-23.

Related Articles. Links



Adenovirus 5 E1a-mediated gene therapy for human ovarian cancer cells in vitro and in vivo.

Zang RY, Shi DR, Lu HJ, Cai SM, Lu DR, Zhang YJ, Qin HL.

Department of Gynecological Oncology, Cancer Hospital and Cancer Institute, Shanghai, China.
ryzang@hotmail.com

The aim of this study was to investigate therapeutic efficacy of adenovirus-mediated E1a gene therapy for ovarian cancer in vitro and in vivo. Recombinant replication-deficient adenoviral vectors were prepared by superinfection of 293 cells, and then purified. The efficacy of the adenovirus vector system to infect ovarian cells was tested using different multiplicity of infection (MOI) and different times (1-4) of Ad.RSVlacZ. SKOV-3 cells (10³ per well) were infected once with 2 x 10⁴ adenovirus. The cells were harvested and counted on different days for 7 days to generate the in vitro growth curve. Tumor-bearing mice were injected intraperitoneally with ovarian cancer cells and treated by intraperitoneal injection of 100 microl (2.5 x 10⁸ PFU) viral solution containing either replication-deficient Ad.E1a(+); control virus Ad.E1a(-) which is the same adenovirus as Ad.E1a(+) except for E1a deletion, or just phosphate buffered solution. The transduction efficacy increased with higher MOI and reached a plateau at the 20:1 ratio. When Ad.E1a(+) was used to transduce the HER-2/neu overexpressing human ovarian cancer cell line SKOV-3, tumor cell growth in vitro was greatly inhibited by E1a transduction. Also, Ad.E1a+ greatly inhibited tumor growth of SKOV-3-bearing mice. Immunohistochemistry analysis indicated that Ad.E1a protein was expressed in tumor tissue and expression of HER-2/neu p185 protein was suppressed. Very strong beta-gal staining was detected in tumors, and beta-gal activity in small intestine, lung, heart, stomach, liver, and kidney was detected. No beta-gal activity was detected in the tumor and other organs in control mice injected with Ad.E1a(-) or PBS. Adenovirus-type 5 E1a gene can efficaciously inhibit HER-2/neu-overexpressing ovarian cancer, and this promising procedure could greatly benefit ovarian cancer patients with high expression of HER-2/neu.

PMID: 11285029 [PubMed - indexed for MEDLINE]